EVALUATION OF HEAVY METALS AND METALLOID ACCUMULATION IN A SMALL-SCALE AQUAPONIC SYSTEM AND AN EFFECT ON BACTERIAL ANTIBIOTIC RESISTANCE

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by ***Hany Mohammad Almotairy***, titled *Metals and Metalloid Accumulation in a Small-Scale Aquaponic System and an Effect on Bacterial Antibiotic Resistance* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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DEDICATION

To the gift of life, my compassionate mother, Fatimah, I dedicate this dissertation to you, and to my pure father soul, Mohammad.

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ABSTRACT

Aquaponics is an environmentally friendly and sustainable technology that holds promise to promote global food production. However, in aquaponics, routine fish feeding, along with the natural phenomena of evaporation and transpiration from the water and plants, could lead to deterioration of water quality of the system and may concentrate organic and inorganic pollutants such as heavy metals (HMs). Aquaponics might present food safety hazards to consumers if they consume food that exceeds maximum allowable limits (MAL) of contaminants and toxins in food and feed set by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO). This work evaluated the presence, diversity, distribution, and accumulation of artificially elevated toxic metalloids and heavy metals (HMs) (arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)) after inoculating them into aquaponics water at levels of 20%, 15%, 1.0%, and 1.0%, respectively, of the U.S Environmental Protection Agency (U.S EPA) standards that dictate the maximum contaminant level (MCL) for drinking water. Work determined the concentrations, distribution, and the fractions of the applied HMs in the water, fish, plant, and sediment. The potential effect of the HMs accumulation on co-selection for bacterial antibiotic resistance (BAR) to ampicillin and tetracycline was also evaluated, and the effect of the HMs on the growth of lettuce, evaluated by root and shoot (edible leaves) elongation was also investigated.

The HMs concentration in the aquaponics water over time were not consistent.

In general, only As accumulated in the water ($P<0.05$), but none of the applied metals exceeded the MCL. The accumulation of As in the aquaponics water may have been due to concentrations of As in fish feed. By the end of the study period (35 days), Hg and Pb tended to bioaccumulate significantly ($P<0.05$) in the fish tissue (wet weight), though both were below the MAL. The root data showed significant ($P>0.05$) accumulation of As, Hg, and Pb, but no HMs bioaccumulated in the shoot of the plant. Unexpectedly, the concentrations of all HMs decreased in the sediment during the final sampling period (the last week), and only Pb was significantly decreased by the end of the trial.

The resistance of bacteria within the aquaponics water to ampicillin and tetracycline showed no consistent patterns. Ampicillin-resistant bacteria decreased in the water over time, while tetracycline-resistant bacteria increased until the third week of the treatment, then decreased gradually until no resistant bacteria were found in samples collected during the final week in the treatment as well as in the control system.

The concentrations of the HMs used in the present work did not have an effect on the root elongation and the shoot length. Also, the plant mass (dry weight) results showed no difference in the treatment compared to the control.

This study can provide a general view of the behavior of HMs in aquaponics, and it can aid the sustainability of the aquaponics industry by ensuring the safety of the products for consumers. Further studies will be needed to shed light on the long-term effects of the HMs (longer period with higher levels).

CHAPTER 1. GENERAL INTRODUCTION

Dissertation format

This dissertation is composed of four chapters representing three studies which have been conducted. These are followed by three appendices prepared as draft manuscripts for submission to professional journals. The three appendices cover: 1) Accumulation of heavy metals in a small-scale aquaponics system; 2) Effect of HMs accumulation on bacterial antibiotic resistance in an aquaponics system; and 3) Effect of some toxic metals and a metalloid on root and shoot length in an aquaponics system. The dissertation's author (Hany Almotairy) was responsible for conducting all experiments described in the manuscripts that are included in this dissertation.

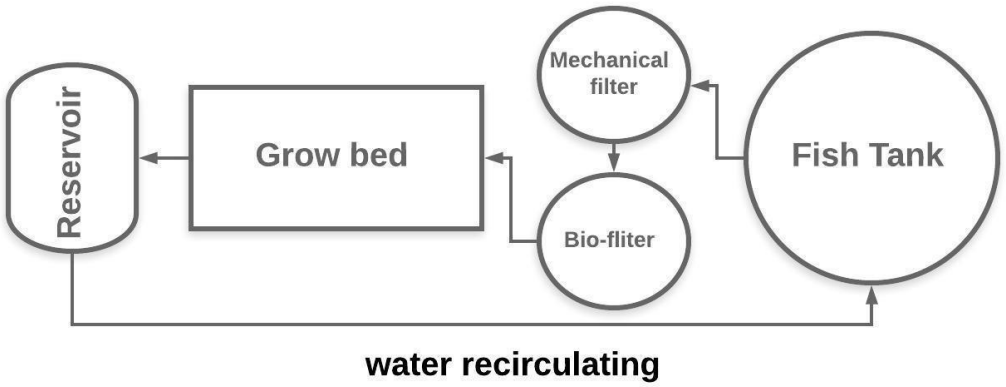
Problem statement

The world population has grown dramatically during the last decade from 7 billion in 2011 to an estimated 7.6 billion in 2019 (UNFPA, 2016). According to the Food and Agriculture Organization of the United Nations (FAO), between 2009 and 2050, global population is projected to increase by over a third, or by an additional 2.3 billion people. With increasing demand for grains for both human consumption and animal feed, grain demand will reach about 3 billion tons by 2050 (FAO, 2009). This highlights an intense need for innovation in agriculture (Touliatos Etal., 2016; Lambin and Meyfroidt, 2011). At the same time, seeking sustainable means to increase food production with consideration for the environment is imperative.

According to the FAO, aquaponics is considered as a future sustainable food production system (Milicic et al., 2017; Wongkiew et al., 2017; Somerville et al., 2014). It is anticipated that this technology can help to alleviate poverty and achieve food security in many developing countries around the world (Bernstein, 2011). The aquaponic concept was developed in the 1970s and its use has become more widespread since that time (Lewis et al., 1978; McBride and Menzel, 1978; Naegel, 1977; Dracup and Mah, 1975; Schneider and Little, 1973). Aquaponic is a modern technology, and a promising method for increasing food yield with less water used, less waste, less labor, low land cost, and fewer chemicals (FAO, 2016).

The word aquaponics is derived from two words: aquaculture and hydroponics (Carlsson, 2013). More precisely, aquaponic can be defined as an integrated method combining “aquaculture” and “hydroponic” systems for farming aquatic animals (mostly fish) and soilless cultivation of plants in a closed-loop recirculated system where fish live with plants in a symbiotic relationship by providing a rich fertilizer effluent for use by the plants. Various functional groups of bacteria metabolize the fish waste into more available forms, e.g., converting ammonia to nitrate. In turn, the plants take up the nutrients to improve the water quality before it recirculates back to the fish again (Fig. 1).

Figure. 1. The concept of Recirculated Aquaponic System.



Assuming low energy costs, aquaponic is a sustainable agriculture method that produces fish and plants together, which increases the economic efficiency of the system (Blidariu and Grozea, 2011). Aquaponics represents an efficient way of producing food using both systems (aquaculture and hydroponics) which can enhance food security around the world. Moreover, especially in developing countries, this technology can aid in overcoming poverty by providing more jobs and mitigating rising food prices, and can also enhance the environment by conserving water and recycling nutrients (Wongkiew et al., 2017). The FAO has recommended and supported aquaponics development by producing a technical manual on small-scale aquaponics food production. The manual serves to encourage the use of aquaponics to enhance food security and use nutrition resources efficiently, which in turn improves the quality of the environment (FAO, 2016).

Reduced availability of fish from the seas, along with the increased demand for seafood, have raised the need for land-based fish farming (Tal et al., 2009).

Aquaculture is the cultivation of aquatic animals under controlled conditions to produce food products in an efficient and less costly way (Ebeling et al., 2006). Aquaculture can provide a vast source of protein; though aquaculture produced only three million tons of fish in the 1970s (FAO, 2018a), currently aquaculture, both in the oceans and inland, provides more than half of all fish for world consumption, contributing significantly to food security around the world (FAO, 2018b). The aquaculture industry is developing rapidly to meet the growing demands for seafood consumption around the world. According to FAO, from 1961 to 2015 fish consumption increased from 9.0 kg to 20.2 kg per capita per year (FAO, 2018b). The global production of fish increased from 70.2 million to 73.8 million tons in 2013 alone (FAO, 2016).

In recirculating aquaculture systems (RAS), water is recirculated between the culture and water treatment stages (Van Rijn, 2013), with less than 10% of the total water volume replaced per day (Blidariu and Grozea, 2011). The use of limited water for substantial seafood production has resulted in the description of RAS as one of the methods for farming of the future (Blidariu and Grozea, 2011).

Hydroponic systems are a culture method that has attracted much attention to produce food in developing countries over the last ten years, especially in areas suffering from water shortages (De Anda and Shear, 2017). The term "hydroponic" was popularized in the early 20th century when scientists learned they could grow plants under soilless conditions by providing nutrients in an aqueous solution (Patil et al., 2016; Chapman et al., 2012). It is an effective alternative method to soil-based agriculture. For example, crops can be grown

where soil is degraded or not available at all (e.g., poor in nutrition or contaminated) such as in urban areas, in polar regions, or in space. The more efficient use of water and enhanced biosecurity reduce disease outbreaks (Benton, 2005). Also, in many situations, hydroponics can increase yield at lower costs (Giro and Ferrante, 2016; Premanandh, 2011) through higher production per unit area, better control of growth, and energy savings (Hosseinzadeh et al., 2017).

Despite these generally positive views of aquaculture and hydroponic, both of these technologies may still have some negative impacts. For instance, current aquaculture is largely seen as a harmful practice to the environment such as organic and inorganic nitrogenous waste (Tal et al., 2009). Also, the production of effluent in aquaculture results in effluent treatment costs. These problems can be turned into advantages when aquaponics is applied, reducing the costs of the individual operation for each component (Wongkiew et al., 2017; Love et al., 2015; Gjesteland, 2013). Due to the high density of fish and protein content of their feed, high concentrations of ammonia–nitrogen are excreted in recirculated aquaculture systems (Wongkiew et al., 2017; Ebeling et al., 2006). In addition to nitrogen (N), dissolved phosphorus (P) discharged into the water contributes to the growth of macro- and microalgae (Rizal et al., 2018). Aquacultural wastes accumulated in the system can impact the rate of production (Buzby and Lin, 2014), and can also impact the environment negatively if discharged inappropriately (Bernardi et al., 2018).

Despite the considerable advantages of a hydroponic system, there are some disadvantages with this method as well. These include the high costs required to

set up the system and the high technical skills commonly needed for operation (Savvas, 2003). Also, there are high expenses for the chemical nutrients required to feed the plants in hydroponics, and the continuous need for replacement of water that has been evaporated and/or transpired (Blidariu and Grozea, 2011). In addition, the wastewater of the nutrient solution drainage from hydroponic systems may be released into the environment, which may cause pollution concerns (Grewal et al., 2011).

Aquaponics can play a significant role by turning the aforementioned disadvantages of aquaculture and hydroponic systems to advantages. With inland aquaculture, water quality needs to be controlled either by a high rate of water exchange or by treating and returning a waste stream; both options are costly. Therefore, aquaponics technology offers an ideal solution to reduce nutrient discharge levels and convert the aquaculture wastes into beneficial nutrients, reducing fertilizer costs for the plants, and can increase profitability by producing two different food products: fish and plants (Rizal et al., 2018; Blidariu and Grozea, 2011). In addition, aquaponics eliminates the need for discharging the concentrated nutrient solution that is often generated in typical hydroponic systems (Bernstein, 2011). Other benefits also may include spreading labor costs between both systems (aquaculture and hydroponic). However, although aquaponics systems have received considerable attention as safe growing systems, there are some potentially serious issues related to human health and the environment; such concerns include HMs pollutants. At present, only a limited number of studies have reported results of HM contamination and

bioaccumulation in fish and/or edible plants in aquaponics systems (Gjesteland, 2013; Rana et al., 2011; Guzel et al., 2018).

A very distinct advantage of aquaponics is in reducing the water needed for raising vegetables (Rakocy et al., 2006). However, evaporation and transpiration are essential processes that influence the water replacement needs for aquaponics (Fang et al., 2017). Nutrient uptake in plants depends on the relatively large amounts of water to transport the dissolved nutrients (Boyer, 1974). In coupled aquaponics systems, most water is lost through the leaves (Gjesteland, 2013). Due to evaporation and transpiration, organic and inorganic pollutants such as heavy metals may be concentrated within the system over time.

Despite the benefits of consuming fish and vegetable crops such as lettuce and tomatoes, such products of aquaponics systems could potentially be sources of bio-accumulated contaminants like heavy metals that pose health risk (Zare et al., 2018; Usydus et al., 2009). Heavy metals can be bioaccumulate over time in fish (Rajeshkumar, and Li, 2018), and plant (Arora et al., 2008) tissues if metals are present in the irrigation water. Fish can accumulate heavy metals in different tissues such as the gill surface, kidney, liver, and gut tract wall through absorption to levels higher than environmental concentrations (Rajeshkumar, and Li, 2018). For instance, tilapia, widely farmed in freshwater aquaponics systems in the United States (Takeuchi and Endo, 2017), have been reported to bio-accumulate metals including lead (Pb), cadmium (Cd), and mercury (Hg) in the edible portions (Abdel-Baki et al., 2011). Vinodhini and Narayanan (2008) showed that various organs of freshwater fish (*Cyprinus carpio)* can become contaminated

when exposed to sublethal concentrations of heavy metals (e.g. Cd, Pb) in water after a period of 32 days.

The physiochemical characteristics of a body of water may limit the availability of an element for plants and may also yield favorable conditions for uptake of a non-essential element (Gjesteland, 2013). Metal toxicity can affect all physiological functions of plants (Barcelo and Poschenrieder, 1990). Metals such as iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) can be bioaccumulated to high levels in vegetables when irrigated with contaminated wastewater. Arora et al. (2008) evaluated heavy metals including Fe, Cu, Mn, and Zn at various levels in vegetables irrigated with wastewater from different sources. The plants accumulated these metals in their tissues to different levels, though concentrations were below the recommended maximum tolerable levels of FAO and WHO (Arora et al., 2008). Cobb et al. (2000) determined uptake and distribution of metals in the edible plant parts (lettuce, radishes, beans, and tomatoes) from a soil contaminated with Pb, Cd, arsenic (As), and zinc (Zn), and found that different plant species accumulated these metals to different levels. Lettuce roots and leaves accumulated similar concentrations of the four metals which may pose risks to consumers. But the long-term effects of accumulation of HMs in vegetables still need to be studied (Graber et al., 2009).

HMs are persistent, and not biodegradable, and their toxicity has become a worldwide problem (Kamika and Momba, 2013). They have long biological half-lives and accumulate preferentially in the body organs (Alkhalaf et al., 2010). The impact of HMs in the ecosystem is not limited to their toxic effects on organisms,

as they play a significant role in inducing bacterial antibiotic resistance (BAR), especially in freshwater ecosystems (Chen et al., 2015; Kamika and Momba, 2013; Holzel et al., 2012; Alexandrino et al., 2011; Altug and Balkis, 2009; Jiang et al., 2008; Dong et al., 1998). Though the mechanism is not fully understood, it is believed that HMs act as co-selection agents, where they play a role in the spreading of antibiotic-resistance genes (ARGs) (Baker-Austin et al., 2006; Ji and Silver, 1995); in other words, metals may act as a selective driver for the spread of BAR and at the same time, antibiotics may act as selective driver for resistance to various metals (Wales and Davies, 2015; Stepanauskas et al., 2006). Berg et al. (2005) found that agricultural soils amended with Cu not only selected for BAR to Cu but also co-selected for resistance to ampicillin, tetracycline, and chloramphenicol. Intensive agriculture practices often include application of fertilizers, pesticides, and sewage sludge, while aquaculture practices include feed additives, all of which can contain metal pollutants such as Hg, Cd, Cu, and Zn that may contaminate soils and water (Cai et al., 2015; Seiler and Berendonk, 2012), increasing the potential for co-selection of BAR in the soil and water of the production environments (Seiler and Berendonk, 2012).

Literature Review

Heavy metals

The term "heavy metal" commonly refers to metals with an atomic number above 20 or with a specific weight higher than 5 g/cm^3 (Lima e Silva et al., 2012; Barcelo and Poschenrieder, 1990), meaning they have a density at least 5 times

greater than that of water (Tchounwou et al., 2012). Heavy metals generally are good conductors of electricity and have common physical properties such as high thermal conductivity, malleability, and ductility (stretches under tensile stress) (Shaw et al., 2004).

Heavy metals contamination can arise from natural or anthropogenic activities. They are naturally occurring and can occur in the crust of the earth as ores of oxide and carbonate, or as sulfides. Anthropogenic sources include industrial and agricultural activities such as smelting, mining, electroplating, industrial discharge, sludge dumping, and certain pesticides and fertilizers (Engin et al., 2015; Ali et al., 2013; Lima e Silva et al., 2012; Shaw et al., 2004), industrial emissions, and automobile exhausts (Dala-Paula Et al., 2018).

Heavy metals are neither created nor destroyed by biological, chemical, or natural processes (Monachese et al., 2012). Therefore, they accumulate in the environment and thus can contaminate the food chain (Ali et al., 2013; Taweel et al., 2013; Lima e Silva et al., 2012). They are often introduced into the environment as co-occurring separate metals or as alloys, and they can attach to small airborne particles. A few metals (e.g., Hg) can exist as vapors (EPA, 2007). Some of the heavy metals such as Cd, As, Pb, and Cr act as carcinogens (Li et al., 2015), and are considered teratogenic and mutagenic agents. In addition, they can be disruptors of the endocrine system and can cause neurological and behavioral changes, particularly in children (Ali et al., 2013).

Some metals are essential for living organisms and are needed in small quantities for normal physiological and biochemical functioning of the cellular

machinery; these essential trace HMs include Fe, Zn, Mn, selenium (Se), nickel (Ni), Cu, sodium (Na), potassium (K), calcium (Ca), Mo, and cobalt (Co). However, even these nutritionally essential metals can be toxic at higher levels (EPA, 2007).

The toxicity of heavy metals to organisms depends on the concentration of metal and chemical form (Bosch et al., 2016). Inorganic metals can convert between inorganic and organic forms and depending on the valence states. The distribution, transformation, absorption, fate, and effects of these metals on organisms depends on the metal compound and the ability of an organism to regulate and/or store the metal (EPA, 2007). In addition, many environmental factors can affect the metal toxicity, such as pH, organic matter content, redox potential, and cation exchange capacity (EPA, 2007).

In water

Heavy metal pollution is a worldwide problem due to their toxicity and their ability to bioaccumulate in living organisms (Ibemenuga, 2013; Taweel et al., 2013; Ikem et al. 2003; Mansour & Sidky 2002). Metals such as Cu and Pb accumulate in freshwater, which poses a risk to human health and the environment (Ali et al. 2013; Salami et al., 2008). According to Aoshima (2012), Cd pollution of the soil in rice paddies of the Jinzu River basin in Toyama, Japan was responsible for widespread occurrence of itai-itai disease.

Natural water usually has low background levels of heavy metals. Eroglu et al.

(2015), report levels of Pb, Cr, Cu, and Zn in natural waters in the range of 0.6–120, 1–10, 0.20–30, and 0.5–10 μg/L, respectively, and levels of Cd <0.1 μg/L. Heavy metals present in sediments can be released into the water, where they may increase to toxic levels (Huang et al., 2017).. Heavy metal toxicity is often a problem in developing countries (Jarup, 2003). For example, heavy metal concentrations have increased to critical levels in some areas of West Bengal due to continuous illegal use of sewage water, unscientific disposal of untreated sewage sludge, and city waste (Saha et al., 2015).

Using heavy metals contaminated water may pose a health risk to humans and the environment. Aquaponics systems may pose more risk of heavy metal contamination in developing countries due to irresponsible activities, lack of knowledge, insufficient regulations, or inadequate commitment to standards.

In fish

Fish represent the main product of aquaponics. Fish contain many essential nutrients such as proteins, vitamins, and minerals (Bosch et al., 2016), and polyunsaturated omega-3 fatty acids (Schenone et al., 2014). In addition, fish contain highly bioavailable forms of essential micronutrients for humans (Golden et al., 2016), contributing to the health of humans around the world (Taweel et al., 2013).

Anthropogenic activities have impacted the aquatic environment for fish (Taweel et al., 2013). Heavy metals such as As and Pb are a significant concern in the aquatic environment due to their toxicity and threat to plant and animal life and for potential human consumption, (Ibemenuga, 2013; Taweel et al., 2013).

Contaminated ecosystems can contribute heavy metals to the tissues of aquatic organisms, where they can bioconcentrate to levels above the ambient environment (Li et al., 2009). High concentrations of these metals in fish tissue can decrease immunity and make fish more susceptible to disease by reducing adaptive ability (Ranasinghe et al., 2016).

Heavy metals can bioaccumulate in fish tissue from the surrounding water through bioconcentration and biomagnification process (Taweel et al., 2013; Lin et al. 2004). Biomagnification of heavy metals occurs over time when higher trophic levels consume lower trophic levels (Ali et al., 2013). Multiple studies have reported on the increasing health concerns of heavy metals due to bioaccumulation in fish tissue (Schenone et al., 2014; El-Moselhy et al., 2014; Abdel-Baki et al, 2011; Eneji et al., 2011; Malik et al., 2010; Vinodhini and Narayanan, 2008; Castro-Gonzalez and Mendez-Armenta., 2008; Uysal et al., 2008Chi et al., 2007; Sekhar et al., 2004; Dural et al., 2007).

Accumulation of heavy metals in fish tissue depends on many factors such as metal concentration, mode of metal uptake, fish age, feeding habits, and environmental conditions (pH, water temperature, salinity, and hardness) (Jezierska and Witeska, 2006). According to EPA. (2007), metal exposure and risks in aquatic environments generally depend on chemical and physical factors such as interactions with natural organic matter, oxidation potential, salinity, and competing with other ions.

Gastrointestinal absorption efficiency and the transference rate are two main factors that play a rule in biomagnification of metals in different fish tissues

(Schenone et al., 2014; Kelly et al. 2008). Metal accumulation shows concentrations in fish follow the following ranking: Fe>Zn> Pb>Cu>Cd>Hg (Jezierska and Witeska, 2006). HMs can be taken into fish tissue through absorption across the gill and surface mucus or through the gut (Ibemenuga, 2013). In fish tissues, the liver, kidney, and gills accumulate metals most strongly, while muscle tissues usually concentrate the lowest levels (Jezierska and Witeska, 2006).

Malik et al. (2010) evaluated the bioaccumulation of heavy metals including Zn, Pb, Cd, nickel (Ni), copper (Cu), chromium (Cr) and Hg in freshwater fish tissues of *Labeo rohita* and *Ctenopharyngodon idella*. They found that different organs accumulated varying quantities of heavy metals. However, despite the accumulation in the organs, the concentrations of heavy metals were within the maximum permissible limits. Feldlite et al. (2008) worked to develop a standard for (As, Cd, Pb, and Hg) in reclaimed water used for edible fish aquaculture for two years. They reported no detectable levels of As and Hg in fish tissue, but levels of Cd and Pb were found above the food standard.

In addition to the physiological effects on fish, heavy metals can also have an effect on the reproductive capacity of aquatic organisms. Burridge et al. (2010), report that sediments enriched in Cu, Zn and Ag had negatively affected clam (*Macoma balthica*) reproduction by reducing gamete production. Reproductive success recovered when metals contamination decreased.

In plants

Plants serve two purposes in an aquaponic system. First, they can be grown for food. Second, they remove chemicals such as ammonia which can be toxic to fish. Plants can also accumulate heavy metals such in the edible portions (Khan et al., 2015; Michalska and Asp, 2001). Several studies have focused on the bioaccumulation of heavy metals in crop plants (Cobb et al., 2000; Khan et al., 2015; Pinto et al., 2004), including lettuce (Smical et al., 2008; Khan et al., 2008; Ahumada et al., 1999).

Accumulation of heavy metals in plants can pose a direct threat to a human health via consumption of contaminated plants or can threaten health indirectly through consumption of food animals raised on a contaminated plant-based diet (Lwalaba et al., 2017). Crews and Davies (1985) showed that humans who consumed vegetables grown in soils with a high concentration of Cd and Pb had blood Pb concentrations 28% higher than others who consumed no local vegetables.

About 400 plant species have been identified as metal hyper-accumulators because they can accumulate metals such as Ni, Zn, or Co to exceptional concentrations after extracting them from the soil into aboveground tissues (Kramer et al., 1997). In addition, some plant species may accumulate specific heavy metals (Engin et al., 2015). Li et al. (2015) measured the concentration of heavy metals (Cr, Ni, Cu, Pb, and Cd) indifferent vegetables at two contaminated sites, and among the plants studied, the highest concentrations of metals in the plants were found in lettuce (*Lactuca sativa L.*) for the first site, and in endive

(*Cichorium endivia*) for the second site. Crews and Davies (1985) report that the uptake process of Cd and Zn into lettuce increased with increasing concentration in the soil.

Roots and shoots are the two main structural components of most vegetables. Water rises from plant's roots through vascular tissues then to leaf cells by capillary action and finally moves to the atmosphere in vapor form by transpiration (Pawar, 2017). Metals may move to the root surface in the soil in two ways; either through concentration gradient and diffusion or by ion exchange between the clay particles and the root (Shaw et al., 2004). The elements can move into the roots by several actions such as passive diffusion through the root cell membrane, active transfer against concentration, or electrochemical potential gradients.

Plants can be accumulators or excluders of heavy metals. Several factors can influence heavy metals uptake into a plant, including pH, oxidation-reduction potential (Tangahu et al., 2011), temperature, aeration, size and type of plant (Shaw et al., 2004), soil organic matter content, and elements availability (DalaPaula et al., 2018). Concentrations of different heavy metals in plants (terrestrial and aquatic) can vary to a large extent. According to Shaw et al. (2004) reported the following values As (0.02-7 µg/g), Cd (0.1-2.4 µg/g), Hg (0.005-.02 µg/g), and Pb (1-13 µg/g) of a dry weight from several studies.

The toxicity of many heavy metals in plants depends on their subcellular distribution and chemical forms (Lwalaba et al., 2107; Zeng et al., 2011).

Elevated concentration of heavy metals can influence the plants' physiological processes, such as water regulation (Santala and Ryser, 2009). Excessive accumulation of Cr can reduce growth and seed germination in plants (Zeng et al., 2011). Excess metal ions can also damage cellular membranes (Barcelo and Poschenrieder, 1990), and can changes a plant's ability to absorb nutrients. This can result in a nutrient deficiency which could have negative consequences in developing countries where populations are already suffering from a lack of nourishment (Khan et al., 2015). Increasing heavy metals concentrations commonly reduce root growth more than shoot growth (Santala and Ryser, 2009), but impacts on shoot growth can still be significant. Burzynski and Klobus (2004) studied the effects of different concentrations of some heavy metals (Cu, Cd, and Pb) on photosynthesis of cucumber leaves (*Cucumis sativus L.*), and in addition to decreasing plant dry mass, water percentage, and chlorophyll content, Fe content decreased as well. Although Cd was more readily transported to cucumber leaves than Cu and Pb, Cu was the most toxic to photosynthesis.

Some metals can cause plant death at levels that are not high enough to be considered toxic (EPA, 2007). Santala and Ryser. (2009), reported that increasing heavy metal levels (Cu and Ni) reduced plant size (birch seedlings) correspondingly at levels ranging from 0% to 2.5% in a growth substrate. Karuppanapandian and Kim (2013) studied the effect of Co on Indian mustard plant (*Brassica juncea L.*) growth in a hydroponic experiment and found that excessive amount of Co in plant tissues caused serious damage to cell membranes, reducing plant biomass and its growth, and eventually plant death.

Generally, the part of the plant that accumulates metals the most is roots (EPA, 2007). Heavy metals inhibit root growth, and this is a common feature of heavy metal stress. Hartley et al. (1999) investigated the effects of soil contamination with single and multiple heavy metals including Cd, Pb, Zn, Antimony (Sb), and Cu on Scots pine seedlings colonized by ectomycorrhizal fungi. Root and shoot growth in a metal-amended soil were both significantly inhibited. Of the metals tested, Cd was the most toxic to the symbiotic relationship of Scots pine seedlings and ectomycorrhizal fungi. Plants often increase their root system size in drought situations if they not exposed to metal stress and thus, HM accumulation in roots can negatively affect uptake of water. In addition, resistance to water flows into and within roots increases.

In sediment

Heavy metals can be absorbed from the water column and deposited on fine surface particles of sediments (Wang et al., 2015; Ikem et al., 2003). In general, their concentrations in sediments are higher than in water (Dummee et al., 2012). Chemical reactions with metals in sediments can change the oxidation state or valence of the heavy metals and subsequently in the water body (Mendiguchia et al., 2006). Sediments of many China reservoirs used as sources of drinking water have been contaminated with heavy metals (Wang et al., 2015).

Due to remobilization of these contaminants in aquatic systems, metals can redistribute from sediments and into the water column and thus into fish (Ikem et

al. 2003). The release of heavy metals from sediments depends on factors such as metal speciation, sediment pH, and water hardness (Ikem et al. 2003).

In fish feed

Heavy metals may enter into animal feed purposely (Eskandari and Pakfetrat, 2014) as they can be added as mineral supplements when they are added in amounts more than required (Alkhalaf et al., 2010). Unintended contamination of animal feed with heavy metals may occur due to several processes, such as irrigation of feed sources with contaminated water, or using contaminated fertilizers or metal-based pesticides (Alkhalaf et al., 2010). Eskandari and Pakfetrat (2014) tested 40 industrial animal feeds in Iran, and they found that 5%, 17% and 42.5% of feed samples were contaminated with As, Cd and Hg, respectively. In each case, concentrations were higher than the MCL.

Fish feeds can be a source of elevated heavy metals in water that did not exist before; it is a crucial factor and primary source of the metals in confined environmental systems (Schenone et al., 2014) such as aquaponic systems. Martins et al. (2011) studied the accumulation of some heavy metals (As, Cd, Pb, and others) in the water of RAS systems with different water exchange rates and reported increased presence of heavy metals in the water, particularly at low water exchange rates. It is likely that these originated from the feed.

Little information exists regarding heavy metals accumulation in aquaponic systems. With the growing use of aquaponics for production of human foods

worldwide, examination of the potential for accumulation of heavy metals in these systems is critical for the protection of human health and the environment.

Arsenic

As is a metalloid commonly found in the Earth's crust and is a member of Group 15 of the periodic table with an atomic number of 33 and an atomic mass of 74.91. Within compounds, As can exist in four different valence states; –3, 0, +3, and +5. Elemental As and arsine (-3) can exist in strongly reducing environments, but under moderately reducing conditions, arsenite (+3) may be the dominant form while arsenate (+5) is generally the stable oxidation state in oxygenated environments. As is present in more than 200 mineral species, occurs naturally in the environment, and one-third of the As in the atmosphere came from natural origins such as volcanic activity (WHO, 2001). Inorganic forms of As from geological origin are the predominant species in freshwater aquatic ecosystems including sediments, surface water and groundwater whereas organic forms such as arsenobetaine, arsenocholine, arsenosugars, and tetramethylarsonium salts predominate in marine organisms, and terrestrial species as well (WHO, 2001; Ohki, and Maeda, 2002). Human activities can produce As compounds as a by-product of metal smelting operations, timber treatment, and burning of fossil fuels. As is also found in agricultural chemicals and pharmaceuticals.

As poses a health hazard to humans when it contaminates drinking water (Shaw et al., 2004). Arsenate, arsenite, methylarsonic acid (MMA) and dimethylarsinic acid (DMA) are forms of arsenic that can be dissolved in the

water column (WHO, 2001). Common forms in drinking water are inorganic As which occurs mainly as trivalent and pentavalent compounds form, As^{+3} and As^{+5}, respectively (Hopenhayn, 2006). As is commonly present in surface freshwaters at concentrations less than 10 µg/liter, concentrations in sediment can range from 5 to 3000 mg/kg. Arsenic is an analog to phosphate, both use the same transporters to cross the plasma membrane of the plant root cell (Abbas et al., 2018) and as such, terrestrial plants can uptake As through roots. In addition, plants can adsorb airborne arsenic deposited on the leaves (WHO, 2001).

Though nearly all As in well-oxygenated water and sediments is present as arsenate (+5), redox potential (Eh), pH, organic content of sediments, and chemical and biological factors affect the interchange of oxidation state. Redox transformation between As(+3) and As(+5) in the environment in response to pH. At a pH=5.8 As(+5) is slightly more mobile than As(+3), and when pH changes from acidic to basic, As(+3) increasingly tends to be the more mobile species (WHO, 2001).

The largest human exposure to As in many human populations comes from seafood (Taylor et al., 2017; Taweel et al., 2013). Organic As is the most common form in seafood; Marine organisms naturally bio-accumulate organic arsenic compounds mostly as arsenobetaine.

In humans, As metabolism has two main reaction pathways: first, reduction of As (+5) to As(+3), and second, oxidative methylation, which allows inorganic As forms to be easily removed from the body in urine (WHO, 2001). Large doses of inorganic As can be toxic to human health causing gastrointestinal effects,

disturbances of cardiovascular and nervous system functions, and death. Exposure to contaminated drinking water for a long time can increase the incidence of cancer in the lungs, skin, bladder, and kidney (WHO, 2001), hypertension, diabetes, neurological disorders, and reproductive problems (Hopenhayn, 2006).

As toxicity impacts on cellular level include chromosomal alterations of cells, generation of oxidative stress, cell proliferation, and inhibition of DNA repair (Schoen et al., 2004). In laboratory animals, inorganic and organic As can cause acute lethality or chronic effects including cancers. Toxicity of As is dependent on the chemical form and the oxidation state. The toxicity of As(+3) comes from its mechanism of binding to protein sulfhydryl groups (WHO, 2001) which are essential to activate various biological responses like platelet activation (Margaritis et al., 2011). As(+5) can affect oxidative phosphorylation (WHO, 2001), a metabolic process that provides usable energy to cells (Fernandez-Vizarra et al., 2009).

Cadmium

Cd occurs naturally in the environment in mineral deposits and can be found widely at low concentrations. Anthropogenic activities such as a manufacture of pigments, metal coatings, plastic stabilizers, batteries, electronics, and nanoparticles used in solar cells (EPA, 2016). Also, from phosphate rock formations (McGeer et al., 2011), Cd can be produced as a byproduct of the smelting of zinc, Pb, or copper ores. Burning of fossil fuels such as oil or coal and municipal waste incineration are primary sources of Cd in the air (EPA, 2000).

Despite its natural presence in the environment, human activities are responsible for about 90% of Cd found in surface waters (EPA, 2016).

Cd is a non-essential element with no known biological function in freshwater ecosystems (EPA, 2016; Wright and Welbourn, 1994). Cd in aquatic systems tends to accumulate in sediments, but under certain conditions, it could be redistributed to the water column (Wright and Welbourn, 1994). In response to Cd exposure aquatic animals and plants produce proteins, called metallothioneins, for binding heavy metals (Wright and Welbourn, 1994). Metallothioneins are low-molecular weight polypeptides present in all eukaryotes and certain prokaryotes, and have a high affinity for Hg, Cu, Zn, and Cd. Thus, their production is induced by exposure to increasing levels of these metals (Wright and Welbourn, 1994). Ionic

Cd^{2+} is the most bioavailable and toxic form to aquatic biota (McGeer et al., 2011; Wright and Welbourn, 1994), while Cd chloride ($CdCl_2$) is most bioavailable in seawater (Wright and Welbourn, 1994).

Factors that can affect the Cd chemical forms include; salinity, organic matter content, hydrogen ion concentration, and Ca ion concentrations (Wright and Welbourn, 1994). Other factors that can have an effect on the availability of Cd for plants include soil organic matter content, chloride concentration, pH, and Cd:Zn ratio (Zare et al., 2018). Cd and Zn can compete with each other to accumulate in edible parts of plants because they are chemically similar (Zare et al., 2018) and thus, Cd uptake by plants depends on the concentrations of HMs in which the plants were grown (Intawongse and Dean, 2006).

Cd is toxic to both humans and animals; short-term exposure to Cd through inhalation can affect the lungs, while chronic oral exposure or inhalation can cause kidney disease (EPA, 2000; Wright and Welbourn, 1994). The kidney is the organ most often affected after long-term exposure to Cd (Aoshima, 2012). The most severe level of chronic exposure to Cd can cause a disease called itai-itai, a disease name given for Japanese people who suffered from Cd poisoning in the 1950s (Aoshima, 2012).

Chronic exposure to Cd has several effects in aquatic organisms such as reduced growth and reproduction as well as impaired immune and endocrine systems while acute exposure causes an elevated mortality rate (EPA, 2016). Commonly, dissolved Cd readily enters through the gill epithelium via channels affinity for Cd estimated to be 100 times higher than that for Ca (Wright and Welbourn, 1994). The concentration of Cd in fish tissues from dietary exposure generally accumulates: maximally in the intestine, then in the liver and kidney, gills, and minimally in muscle (Le Croizier et al., 2018). In fish, acute toxicity results in disruption of ion homeostasis while chronic exposure can disrupt endocrine and ionic regulations, and can inhibit immune system function, and stunt growth (McGeer et al., 2011).

Cd can accumulate in the edible parts of plants, which poses a potential hazard for both human and animal health; Cd uptake from vegetables and cereals is responsible for more than 70% of human exposure (Michalska and Asp, 2001). Within plants, Cd effects can be found at cellular level, e.g., membrane damage and enzymes inhibition/activation; other effects include seed germination

inhibition, impaired respiration and transpiration, reductions in growth rate, and induction Fe deficiency (Lopez-Millan et al., 2009).

Crops grown in contaminated soils are considered as a primary source of Cd in humans (Zare et al., 2018). In general, for non-smokers, food including leafy vegetables such as lettuce (*Lactuca sativa L*.) and rice (*Oryza sativa*), is the main exposure source of Cd. For smokers, tobacco grown in contaminated soil is the major source (EPA, 2000; Lopez-Millan et al., 2009). Cd tends to accumulate to higher levels in leafy vegetables (Chaney et al., 2001); Dala-Paula et al. (2018) evaluated some heavy metals (Cd, Cu, and Pb) in lettuce and soils from urban locations in Brazil and found, in addition to a potential risk for Cd accumulation, there existed a significant relationship between Cd in lettuce and in the soil. Lopez-Millan et al. (2009) studied the effects of Cd in tomato (*Lycopersicon esculentum*) grown in a hydroponic system, and they found that low Cd levels (10 µM) may induce a moderate Fe deficiency while high concentration of Cd (100 µM) affects plant growth and the ability to utilize Fe. In addition, excess Cd had effects on photosynthetic rates and concentrations of photosynthetic pigment.

Mercury

Hg is the only metal which is liquid at ambient temperatures (Kidd and Batchelar, 2011; CDC, 1999), and it is a non-transition metal located in group number 12 of periodic table. It has low melting and boiling points, (-38.9°C) and (356.58°C) respectively with higher volatility comparing to other metals (Kidd and Batchelar, 2011). There are four main oxidation states for Hg (Hg0, Hg^{+1}, Hg^{+2}, and Hg^{+3}). It occurs naturally in Earth's crust and is distributed in the

environment by natural and human activities; natural sources include weathering of soils and rocks, volcanic eruptions, and forest fire (Kidd and Batchelar, 2011; Crump and Trudeau, 2009). Anthropogenic activities include emissions from fossil fuel combustion and waste incineration, as well as gold mining and smelting. Hg is also used in pigments, chloralkali process (producing chlorine and sodium hydroxide), scientific instruments, preservation of vaccines, pesticides (Kidd and Batchelar, 2011), and illumination and electronic products (Tsydenova et al., 2011).

There are several factors that can affect the bioaccumulation of Hg in fish tissue: speciation of Hg, methylation and de-methylation rates, the concentration of dissolved organic carbon and interactive effects among these factors (Grieb et al., 1990). Methylmercury (CH_3Hg^+) is the organic form of metallic Hg, and it can affect human and animal health (Kidd and Batchelar, 2011; Harris et al., 2007). Microscopic organisms, mainly sulfate-reducing bacteria, in water and soil can produce CH_3Hg^+ through a methylation process; thus, increases in environmental concentrations of Hg may also increase CH_3Hg^+ (CDC, 1999).

Methylmercury is the primary form of Hg found in freshwater and marine fish (Rolfhus and Fitzgerald, 1995). Because it be bioaccumulated in fish tissue, larger and older fish may have higher concentrations (CDC, 1999).

In freshwaters, the two main inorganic forms Hg^0, Hg^{2+} (Kidd and Batchelar, 2011) generally have low water solubility, but the solubility increases when complexing with dissolved organic carbon an Hg can evaporate from the water surface naturally (Penman, 1948) and its emissions from surface waters in both

marine and freshwater systems have been widely reported (Lindberg et al., 2000). Although the mechanism of Hg evaporation from water may be based biologically, recent evidence suggests that direct photochemical reduction of Hg^{2+} species to dissolved metallic mercury vapor (Hg^0) may play a significant role (Lindberg et al., 2000).

Increased concentrations of Hg pose health risks for human and wildlife worldwide (Crump and Trudeau, 2009). Hg can cause heart, kidney and lung disease, changes in vision or hearing, memory problems, and brain damage (Skinner et al., 2007; CDC, 1999). Fish development and growth can be affected by the toxicity of Hg (both Hg^{2+} and CH_3Hg^+) from water (Kidd and Batchelar, 2011) and food sources (Crump and Trudeau, 2009). In general, inorganic Hg is the most toxic form to nerve tissues of fish (Crump and Trudeau, 2009) and exposure to high concentrations can cause respiratory distress, and ultimately death (Kidd and Batchelar, 2011). Generally, at lower concentrations, Hg may indirectly affect fish populations by influencing reproductive organs, including reductions in gonad size, gamete production, and circulation of reproductive steroids (Crump and Trudeau, 2009). Bio-accumulation of CH_3Hg^+ can affect their behavior, swimming activity, starvation, growth, and mortality (Crump and Trudeau, 2009).

The mobility and uptake of Hg in soils is low (De Temmerman et al., 2009). Barker (1972) studied toxicity levels of Hg along with other metals (Pb, Cu, and Zn) on tissue cultures of different crops (lettuce, cauliflower, potato, and carrot).

The results showed that lettuce growth was inhibited with concentrations less than 0.005 mg/liter. At 5.0 mg/liter, the plant was nearly dead, with gray-colored leaf tissue. In general, Hg is available to plants only in very limited amounts; in large quantities, most of the Hg remains in unavailable forms bound to soil clay and organic matter (Cappon, 1981).

Lead

Pb is a divalent cation (Needleman, 2004). It is found naturally in the Earth's crust in ores that include other elements such as Ag, Cu, and Zn. The primary source for releasing Pb to the environment is anthropogenic activity. It has become one of the most widely distributed metals worldwide due to its features: it is highly malleable, ductile, and easy to smelt which promotes its use for building construction, weights, fusible alloys (Cheng and Hu, 2010), printing, pigments, petrochemicals, fossil fuel combustion, photographic materials, glazes, solder, plastics, and car batteries (Zahra, 2012). In addition, mining, smelting, coal burning, garbage incineration, and addition of Pb to paint, pesticides, and gasoline have increased pollution in the environment (Cheng and Hu, 2010).

It is believed that 20% of total Pb exposure in the U.S. is via drinking water (Triantafyllidou and Edwards, 2012). Physicochemical speciation of Pb controls its solubility in drinking water and the effectiveness of absorption from the gastrointestinal tract (Harrison and Laxen, 1980). In general, Pb is present in the environment in inorganic forms and can exist in different oxidation states (0, +1, +2 and +4). Pb oxidizes in drinking water, and is found in +2 valence state

(Schock, 1990) which is the most stable ionic form that can bioaccumulate in aquatic organisms. The primary pathway of uptake of Pb^{2+} in fish is by the gills then into the bloodstream (Ahmed and Bibi, 2010).

Pb has carcinogenic, teratogenic, mutagenic, genotoxic effects to humans and animals (Winiarska-Mieczan et al., 2015). It can accumulate in bones, brain, and muscles and may cause disorders including anemia, kidney diseases, nervous system disorders, and death. Chronic exposure to Pb^{+2}, even at low levels, can cause damage to the nervous and digestive systems skeleton (Zahra, 2012), kidneys, blood pressure regulation (Winiarska-Mieczan et al., 2015), brain development, and may cause behavioral disorders (Needleman, 2004). It can enter the human body from contaminated sources through inhalation and ingestion (Cheng and Hu, 2010). Lead can bind strongly to sulfhydryl groups on proteins, and its toxicity comes from the ability to compete with Ca in the body cells (Needleman, 2004).

Pb is carcinogenic to fish and can negatively affect reproduction, liver and thyroid function, and prolonged exposure to high levels of Pb can induce muscular and neurological effects, growth inhibition, paralysis, and mortality (Ahmed and Bibi, 2010). The concentration of Pb within the fish depends on many factors including eating habits, doses, routes of absorption, and excretion average (Winiarska-Mieczan et al., 2015). Acute exposure to Pb^{+2} can disrupt the fish immune system and kidneys functions while high concentrations (100 to 200 g/day) can cause brain damage (Zahra, 2012).

Although Pb is not known to be an essential element for any plant, it may enter the food chain in the edible parts of plants (Michalska and Asp, 2001; Chopra and Pathak, 2012). Pb has toxic effects of plant growth, and roots concentrate more than leaves (Broyer et al., 1972). The accumulation may occur due to the low mobility of Pb in soils and the long residence time in the environment (Uzu et al., 2010). The accumulation of Pb into several plants has been documented, including as tomato and eggplant (Khan and Khan 1983), and lettuce (Michalska and Asp, 2001; Uzu et al., 2010; Beavington, 1975; Sterrett et al., 1996).

Bacteria

Bacteria play a significant role in aquaponic systems where fish and plants grow symbiotically. Nitrogen (N) is an essential component of living organisms because it is a major part of building blocks of all proteins (amino acids) and genetic material (nucleic acids) (Khakyzadeh et al., 2015; Wongkiew et al., 2017). Fish excrete ammonia into the water where nitrifying bacteria convert it to a potentially toxic form, nitrite (NO_2^-), which has an extremely high affinity to bind with hemoglobin and thus decrease the oxygen availability for cell respiration. Bacterial nitrification continues by converting NO_2^- to nitrate (NO_3^-) which is not toxic and is a form of N that most plants easily assimilate (Thurmer, 2014; Wongkiew et al., 2017).

There are many parameters which strongly affect nitrification process. Thus, to ensure the water quality of aquaponic systems, humidity, air and water temperature, pH, dissolved oxygen (DO), and photosynthetically active radiation

(PAR) should be monitored.

Heavy metals and bacteria

Some bacteria have a tolerance to heavy metals toxicity, and can be used to bioaccumulate, and thus recover these metals from sources such as mine tailings or from contaminated environments where they may receive industrial effluents (Lima e Silva et al., 2012; Gupta et al., 2012). For example, different bacteria species such as *Pseudomonas* can be efficient in the bioaccumulation of several heavy metals like Cu, Cd, and Pb and other metals ions that may presented in the polluted effluent (Malekzadeh et al. 2002).; beside, elect aerobic microorganisms (bacteria, fungi, and yeasts) can accumulate metals such as Cd, Ag, Co, Cr, Ni, and Cu; therefore, they are used for bioremediation processes. These microorganisms have evolved strategies to overcome heavy metals toxicity; they can transform the metal to a less toxic form, or by binding the metal inside or outside of the cell which can protect the host cell from the interaction with the heavy metal to avoid harmful effects (Monachese et al., 2012).

Due to the net negative charge of bacteria (cell surface or cell wall) and the cationic charge of many heavy metals, the heavy metals bind to the cell wall of some bacterial species (Monachese et al., 2012). However, other species of bacteria can be negatively impacted by the elevated levels of heavy metals in the environment (Zampieri et al., 2016). The toxic effects of heavy metals on microorganisms are influenced by a multitude of factors such as pH, chelating agents, concentration, speciation, and organic matter (Lima e Silva et al., 2012).

Present Study

To improve food production, minimize environmental impacts, and promote sustainable development of aquaponic systems, it is necessary to identify the potential risk of heavy metals accumulation in the systems and identify any health hazard concerns of BAR due to the increased concentration of some heavy metals in the system. Studying these issues in aquaponics will help researchers, food safety specialists, and aquaponic growers, particularly in developed countries, to determine the effects of metalloid and heavy metals accumulation on fish and plants in aquaponic systems and their effects on enhancing the BAR.

The experiment was conducted in the University of Arizona, Controlled Environment Agriculture Center (CEAC) at greenhouse #3118 for 35 days from February to April 2018. The aim of this study was to investigate the distribution of four toxic metalloid and metals elements (As, Cd, Hg, and Pb) in components of the aquaponic systems (water, fish, plant, and sediments) at near-zero makeup water, and to study the effect of these pollutants on development of BAR.

The design of the experiment included six replicates: three control, and three representing the treatment, with HMs spiked into the water of the replicates on the first day at target concentrations of 20%, 15%, 1.5%, and 1.0%, respectively of the MCL of the U.S. EPA standards for drinking water quality (EPA, 2018). International food standards set by the joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives

(FAO/WHO, 1995) were considered as the MAL available of (CH_3Hg^+) for fish, and both Cd and Pb for the lettuce (as leafy vegetable standards) based on wet weight of the samples. All replicates were stocked with 25 fingerling tilapia fish (*Oreochromis niloticus*) (25–50 g) provided by "Desert Springs Tilapia, Dateland, AZ, U.S. and six butterhead lettuce (*Lactuca sativa*) plants from Johnny's Seeds, Winslow, ME. The hydroponic system used in this experiment is considered a deep-water culture (DWC). Tap water was used and commercial fish feed was fed (AquaXcel starter 5014 0.8 mm diet, with 50% minimum crude protein, 14% minimum crude fat, 2% maximum crude fiber, and 1% minimum phosphorus) from Cargill Animal Nutrition, Casa Grande, AZ.

Statistical significance ($P < 0.05$) between the treatment and the control were determined using t-tests for equal n. Six weeks of water data, and data for three weeks of the sediments (week 3 to week 6) were analyzed while the data of the fish and the lettuce (root and shoot) were analyzed by comparing the first day of the experiment with the last day.

The study included three hypotheses. First, HMs will be concentrated to a higher level in components of the spiked treatment than the control. Second, Bacterial antibiotic-resistance to ampicillin and tetracycline will be significantly greater in the treatment spiked with HMs than the control. Third, roots and shoots exposed to HMs will be significantly shorter than controls

Although As accumulated in the water of both the control and the treatment over the 35-day experiment, all HMs concentrations were within the MCL (10 µg/l) except for the second treatment of As ($P < 0.05$) which exceeded the MCL

(10.06 µg/l) by the final week, while As levels of the other replicates ranged from 8.3- 9.5 µg/l.

Based on the food standard MAL by FAO/WHO, among the metals tested, only Pb and Hg as CH_3Hg^+ have standards as wet weight (µg/g). Although both Pb and Hg accumulated in the fish tissue ($P < 0.05$) by the end of the experiment compared to the control, their concentrations were still well below the MAL. HMs accumulation in the fish tissue as dry weight showed that only Hg and Pb ($P < 0.05$) accumulated in both the treatment compared to the control over the length of the experiment.

MAL's of leafy vegetables are available only for Cd and Pb based on the wet weight (µg/g). These standards apply only to the edible parts of lettuce not the root. Cd did not accumulate in the shoot. Pb concentrations slightly increased in all samples, but were well within the MAL. There were no differences between the treatments and the control among all HMs ($P > 0.05$).

The analysis of the levels of the HMs in the sediment samples showed decrease in all HMs over the last three weeks of the experiment except for the Pb were increased significantly ($P<0.05$).

The results of this experiment reveal no potential health risks for humans at the levels of HMs tested in these systems. However, because As levels in the water exceeded the MCL for the one treatment replicate, further studies may be needed on the accumulation of HMs, particularly As, over longer periods of time in future experiments.

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APPENDIX A - EVALUATION OF ACCUMULATION OF HEAVY METALS IN A SMALL-SCALE AQUAPONIC SYSTEM

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ABSTRACT

Aquaponics can be applied to improve the water quality of aquaculture farms through the culture of fish and plants (hydroponic) in a recirculating water system. It is a technology that holds promise to enhance global food production. However, in aquaponics routine fish feeding along with the natural phenomenon of the water and the plants such as evaporation and transpiration could lead to deterioration of water quality of the system and may concentrate organic and inorganic pollutants such as heavy metals (HMs). Aquaponics might present food safety hazards to consumers if they consume contaminated food that exceeded the standard limits of any toxic element or compound. This experiment evaluated the presence, diversity, distribution, and accumulation of artificially elevated toxic metalloid and heavy metals (HMs) (arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)) in a small-scale aquaponic system to determine the concentrations, distribution, and the fractions of the HMs in the water, fish, plant, and sediment, and evaluating the health aspects of the water, fish, and the plant samples based on the drinking water standards as maximum contamination limits (MCL) of the United States Environmental Protection Agency (EPA) and the maximum allowable limits (MAL) of FAO/WHO as wet weight samples for Hg (methylmercury) and Pb in the fish, and Cd, and Pb for the edible part of the lettuce. Different levels of the HMs were inoculated in the water (the treatment), and the control did not intentionally receive any HMs. Three replicates were prepared for each treatment, and each replicate was stocked with 25 Tilapia and 6 lettuce plants. The experimental treatment received mixed HMs aliquots with As,

Cd, Hg, and Pb at approximately 20%, 15%, 1.5%, and 1.0% levels, respectively based on the maximum contamination level (MCL). In addition to weekly water and sediment samples, fish and plant (the shoot and root) samples were collected on the first day, and on the last day of the experiment to determine the comparison of the HMs concentrations.

There were significantly differences for the levels of As and Hg compared with the control. However, only As accumulated in the water while Hg decreased. No metal exceeded the MCL, but As, as average, reached to a high level with a significant difference compared to the control ($P<0.05$). By the end of the trial period (35 days), Hg, and Pb tended to bioaccumulate (wet weight) significantly ($P < 0.05$) in the fish tissue. Hg and Pb levels in fish tissue of the treatment samples were way below the MAL (20.91 per 500 ng/g) and (73.79 per 300 ng/g), respectively. All the HMs accumulated significantly in the root ($P<0.05$.) except for Cd ($P>0.05$). Unexpectedly, All the HMs decreased in the sediment at the last week; the only difference recorded between the treatment and the control was in the Pb.

Introduction

Aquaponics is a system that combines and integrates recirculating aquaculture system (RAS) and hydroponic systems in one closed-loop environment (Delaide et al., 2016). Aquaponics has succeeded economically in many parts of the world (Gjesteland, 2013), and it's a cost-effective, and environmentally friendly technology (Mangmang et al., 2016). Aquaponics can be used for increasing food yield with efficient water use, less chemical use, less labor, and efficient low land costs (FAO, 2016). However, potential accumulation of harmful contaminants (organic and non-organic) such as heavy metals in tightly closed aquaponic systems is should be examined (Gjesteland, 2013).

Heavy metals are persistent, and they are non-biodegradable, and their toxicity has become a worldwide problem (Kamika and Momba, 2013); they have long biological half-lives and accumulate potentially in different body organs (Alkhalaf et al., 2010). Evaporation and transpiration are essential processes that influence the water exchange in aquaponics (Fang et al., 2017). Aquaponics is a closed-loop recirculated system, and due to evaporation and transpiration processes, organic and non-organic pollutants, such as heavy metals, may concentrate within the system over time. The concentration of the heavy metals in the system may depends on the background levels of the metals in the water source and the concentrations in the fish feed as well as the time of running the system. A pilot study was completed before conducting the current study; the tested HMs accumulated in different parts of the system (water, fish, and the plant) over the period of the trial, and only Cd in a treatment exceeded the

FAO/WHO standard. In a study done by Deviller et al (2005), after one year of culturing fish (European sea bass) in a recirculating aquaculture system (RAS), a reduction (15%) of the final fish weight were attributed to accumulated of different concentrations of several metals such as (As, Cd, Pb, Cu, Zink (Zn), and Cr). However, they were below FAO/WHO standards for the human consumption. Also, in a recirculating aquaculture system (RAS), Martins et al. (2009) studied the influence of several parameters including heavy metals of two treatments with different water exchange rate (30 and 1500 L/kg feed/day) on the embryonic and larval development of carp fish. Metals studied in this trial; As, copper (Cu), manganese (Mn), nickel (Ni), and Zn were significantly higher in the low exchange rate (30L/kg feed/day) while others; lead, iron, and chromium were below detection limit. On the other hand, Davidson et al. (2011) evaluated the health of rainbow trout *Oncorhynchus mykiss* in two trials using replicated water recirculating aquaculture system under different water exchange and feed load rates. In the first experiment, low and near-zero water exchange, and relatively high feed rate were used, but high water exchange and low feed rate used in the second experiment. The fish mortality increased, and deformities with unusual swimming behaviors observed in the first trial. The authors expected that accumulating of potassium and nitrate nitrogen were probably the reason of the adverse fish health and welfare problems, but not the heavy metals (As, Cd, Hg, Pb, chromium (Cr), and others) which were below the minimum detection limit within of the culture water.

Generally, ingestion of inorganic compounds via food or water are the main pathway into the organism. For instance, when ingested As, absorption takes place in the stomach and intestines then passing into the blood (Munoz-Olivas and Camara, 2001). Heavy metals can be present in sediments, water, and organisms (Huang et al., 2017). Studies on heavy metals in aquaponics are scarce. A few studies utilized aquaponic systems to study water quality, heavy metals contamination and bio-accumulation in fish and/or crops such as Nile Tilapia (*Oreochromis niloticus*) and green pepper (Eissa et al., 2015), lettuce (Gjesteland, 2013), tomato (Rana et al., 2011), and cucumber (Guzel et al., 2018). Heavy metals are found in the environment, but their levels are elevated and distributed in the environment due to the human activities increasing the possibilities to reach groundwater or crop plants through contaminated water irrigation (Alkhalaf et al., 2010). Surface and ground water can be naturally contaminated with some heavy metals/metalloids such as As (Schenone et al., 2014), and Pb (Mager, 2011). For example, up to 20% of the U.S total Pb exposure comes from drinking water consumption (Triantafyllidou and Edwards, 2012). Heavy metals also enter into animal feeds (Eskandari and Pakfetrat, 2014) when Cu, Zn, Fe, Mg (essential metals) also get micro-amounts of heavy metals as mineral supplements (Alkhalaf et al., 2010; Burridge et al., 2010) and be a primary source of the metals in confined environmental systems (Schenone et al., 2014).

Metals such as Cu and Pb can accumulate in freshwater (Salami et al., 2008). Fish (Rajeshkumar, and Li, 2018), and plants (Arora et al., 2008) can bioaccumulate heavy metals when exposed over time. Heavy metals can be

biomagnified in fish tissue (Taweel et al., 2013; Ikem et al. 2003; Mansour & Sidky 2002) at levels higher than environmental concentration (Rajeshkumar, and Li, 2018). For instance, tilapia fish bio-accumulate metals such as Pb, Cd, and Hg in the edible fillet (Abdel-Baki et al., 2011). Gjesteland. (2013) studied the quality changes of wastewater from smolt (young salmon) production with a small-scale recirculating system used to grow lettuce for commercial use. The study found that EC levels increased with time as the water reservoir decreased due to water uptake by the plants and evaporation. Intensification, high fish feeding rates, and low water exchange rates in RAS may accumulate substances like heavy metals in the water and fish.

Plants absorb and discharge large amounts of water through the transpiration mechanism (Boyer, 1974). Heavy metals such as Cd and Pb can accumulate in the edible parts of plants as they are left behind when water is transpired (Michalska and Asp, 2001). Plant species have different capacities in accumulating and storing heavy metals, and some plant species may accumulate specific heavy metals (Engin et al., 2015). Lettuce is a metal accumulating plant and Li et al. (2015) determined the concentration of some heavy metals (Cr, Ni, Cu, Pb, and Cd) in five types of vegetables, and among the plants studied, the highest concentrations of metals were found in lettuce leaves. Generally, the portion of the plant that most accumulates metals is the root (EPA, 2007)

The toxic metals As, Cd, Hg, and Pb are harmful to humans, fish, and plants. Therefore, studying the accumulation of these metals in aquaponics is necessary

to determine the effects on fish and plants and as a result, protect human health and the environment as well.

Materials, parameters and instruments

Each replicate has two buckets, one for a mechanical filter and one for a bio-filter. The mechanical filter was used for filtering particulates and settable solids from the water and trapped the sediments. The bio-filter was used for encouraging the nitrifying and heterotrophic bacterial activity; both filters have plastic bioballs as media used for increasing the surface area for bacterial films and enhancing their metabolic activity. The bio-balls provided after old aquaponics system which were stored in the same greenhouse where the study done).

The source of the water used in the system is tap water. System on all replicates was run empty (no fish or plant) for about two days to eliminate the chlorine residuals. The fish were fed for two days to establish and acclimate the system before the experiment started. During the trial, the fish were fed two times a day at 2.5% of biomass until the last day of the experiment. Fish were fed 2.5% of initial biomass for the entire trial. Each replicate contained about 244.1 L of water volume (65 L, 37.6 L, 25.2 L, and 115.8 L in the reservoir, the bio- and mechanical filter, the plant growing bed, and the fish tank, respectively). Due to evapotranspiration and water accumulation in growing biomass of lettuce the replicates received between 20 and 50 liters of de-ionized replacement water during the trial.

Several tools, instruments, and test kits were used for monitoring the water quality. Dissolved oxygen and water temperature were determined with a Yellow

Springs Instrument (YSI) (550A). A Hach Co. DR/890 Portable Colorimeter was used to determine Ammonia Nitrogen (NH_3-N) (salicylate method 8155) and Nitrate (NO_3^--N) (cadmium reduction method 8039). A portable pH meter from Hach Co. (HQ40d) was used for measuring pH, using magnetic stirrer for more accurate results. An electrical conductivity meter (EC) from OAKTON Instruments was used to determine total dissolved solids.

Relative humidity (RH), air temperature (T_a), and photosynthetically active radiation (PAR) ($\mu mol \cdot m^{-2} \cdot s^{-1}$) were also recorded with an automated system in the greenhouse.

Other materials used include; syringe filters (Nylon, 25mm diameter, 0.45um) provided from (Scientific Strategies. Co), and Plastic Syringe (Luer Slip, 10 mL) from (Materro LLC) for preparing water samples prior to submitting for HMs analysis, Grodan rock-wool medium used for establishing the lettuce plants, a forced air drier for drying fish and plant samples prior to HMs analysis, polyester air filter media used in the mechanical filter, plastic plant pots, digital lab scale and analytical scale for measuring weights of samples (wet and dry) and HMs prior to inoculating, ceramic mortar and pestle for grinding the samples (fish and plants), pipettes, paper bags, de-ionized water (Diw), distilled water (Dsw), sterile plastic tubes (15 ml, and 50ml), and Petri dishes (100mm x 15mm).

Chemicals (heavy metals inoculation)

At the beginning of the study, the fish feed and the water used in the experiment were analyzed to determine HMs concentrations (Cd, Pb, Hg, and

As). The analyses for these heavy metals were performed by the Arizona Laboratory for Emerging Contaminants (ALEC) to determine the level of each metal tested (background levels). HMs were spiked into the treatment replicates at the following HMs concentrations (As, Cd, Hg, and Pb) 20%, 15%, 1.5%, and 1.0%, respectively, of the MCL of the U.S Standards for Ground Water and Drinking Water (EPA, 2018). The HMs compounds used were: (As) as sodium hydrogen arsenate heptahydrate (Na_2HAsO_4), (Cd) as cadmium acetate dehydrate ($(CH_3CO_2)Cd{\cdot}2H_2O$), (Hg) as methylmercury chloride (CH_3HgCl), and (Pb) as lead (Pb^{2+}) acetate ($C_4H_{10}O_8Pb_3$). All compounds of the HMs were procured from (Thermo Fisher Scientific Chemicals Inc).

The amount will be needed to be add into the water of the system is based on the MCL targeted for each one of the HMs (µg/L). By knowing the background level of the metal in the water from ALEC (µg/L), the incomplete amount will be added (to one liter) to reach to the target of the metal. Then, multiplying this amount by the total volume of the water in the system to get to the amount we need to add of the metal. Therefore, after receiving the background level of each one of the HMs in the tap water (µg/L), and based on the MCL, we calculated the amount of each metal that we need to add. To do this, we calculated the fraction (weight) of the metal in the container (the bottle) by dividing the molecular weight (MW) of the metal (periodic table) from the total MW (the label of the container). Then by knowing the total volume of the water in the system (a replicate), we knew how much amount of a metal will be added. Each one of the HMs weighed using an analytical balance then diluted in one liter of de-ionized

water, and finally inoculate it into the system (replicate). Concentration of the HMs provided in (Table. 1) as MCL, PPM, and µM.

Table. 1. The maximum contamination level (MCL) of the heavy metals and the metalloid tested in the study and their concentrations that inoculated into the treatment systems in a different units (ug/L, ppm, and µM).

Metal	MCL* (µg/L)	Spiked concentration of the MCL	Targeted concentration (µg/L)**	(ppm)	Concentration in mole (µM)
As	10	20%	7.96	0.008	0.11
Cd	5	15%	1.76	0.002	0.02
Hg	2	1.5%	0.038	4×10^{-5}	0.002
Pb	15	1.0%	0.62	6×10^{-4}	2.9

*Maximum contamination level set by the U.S. Environmental Protection Agency (EPA) (EPA, 2018).

** As as (Na_2HAsO_4), Cd as ($(CH_3CO_2)Cd \cdot 2H_2O$), Hg as (CH_3HgCl), and Pb as ($C_4H_{10}O_8Pb_3$).

In addition to the MCL of the HMs in the water, concentration of the HMs (Hg and Pb) in fish tissue, and (Cd and Pb) in the edible part of the lettuce (as wet weight) were compared with the maximum allowable limits (MAL) set by the FAO/WHO for ensuring safety of consumers (Table. 31).

Fish source

Three hundred juvenile Tilapia fish (*Oreochromis niloticus*) were provided by Desert Springs Tilapia, a commercial fish farm located in Arizona, U.S. The fish were acclimatized for two days. Mortality through the trial was less than 5% of the population. Water temperature was controlled at 24°C during acclimatization.

Seedling and transplanting

Eighty lettuce were seeded nine days before the acclimation period at the start of the experiment. Rock-wool cubes were used for seeding after being

trimmed using a plastic knife. Six plant samples were selected for initial HMs analysis to determine baseline concentration. Then 36 of the remaining plants were randomly selected for transplanting to the six replicates at the start of the acclimation period. Wet weights were determined for baseline samples and the transplanted lettuce plants.

Fish feeding

Fish were fed two times daily based on the average body weight (2.5%) of all fish in the replicates. Therefore, 8.4 g of feed was added daily (4.2 g in morning and 4.2 g in afternoon). The feed ratio was based on the following formula:

$$\text{Feed Ratio} = \left(\frac{Total\ fish\ weight}{Number\ of\ fish\ calculated} \times 0.025 \times \text{Number of fish in the tank} \right)$$

Fish distribution

On the first day, and before stocking any fish in the replicates, six randomly selected fish (13 g total average ± 6 g) were isolated and removed from the original population using a fish net to be the baseline fish samples. The next day, 150 fish (13.5 g average) were weighed (wet weight) and randomly distributed into the replicates, 25 per system.

Plants distribution

The plants seedling for nine days before starting the experiment and they irrigated daily with the tap water. On the day one of the trial, the plant that has not three to four true leaves excluded. Six lettuce plants randomly selected and sorted

into six groups where these groups randomly selected later to be transplanting into the replicates.

Samples

Preparation

The samples collected to track the partitioning and fate of HM's (rock-wool, fish feed, water, fish, plant, and sediments) were prepared in the following manner: rock-wool was cut into small pieces to fit into two 50 ml sterile plastic tubes (one with de-ionized water and the other with distilled water).

Water samples were collected weekly into 15 ml sterile plastic tubes after filtration using a syringe filter (Nylon, 25mm diameter, 0.45um) and plastic syringe (Luer Slip, 10 mL).

Initial condition (baseline) fish and plant samples were collected at the start of the trial. Whole fish samples (euthanized by chill killing using ice water slurry) and lettuce plants transferred, at the first day, using a plastic forceps and then were put on Petri dishes for drying by transferring to an oven at (105°C), and (65°), for the fish and the plant samples, respectively. All samples were kept in ovens until reaching a constant dry weight. The plastic forceps rinsed with dilute nitric acid (HNO_3) with distilled water before and after the use with each sample. [Note: Additional information on the nitric acid (HNO_3) diluting can be found on page 137]. Before drying, each fish sample was rinsed using de-ionized water before taking the wet weight and then dried. Plants were not rinsed. For the end of trial final sampling, fish were treated in the same manner but final plant samples

(only shoot) were collected and stored in individual paper bags before drying after separating from the root. The shoot separated from the root by cutting 0.50 cm from the surface of the rock-wool cube using clean plastic knives; the root removed and isolated carefully using the plastic forceps by opening the rock-wool with hand.

Fish and plant samples were then ground to a coarse powder using a stainless-steel grinder and ceramic mortar and pestle prior to submission to the ALEC. In other studies, fish samples such as muscle, liver, and kidney can be collected as separate or as single samples, but we collected the whole fish carcasses due to the small size of the fish and also to estimate the metal concentration in all organs after homogenizing. The fish samples were homogenized using the blender for 30 sec. Dry weight of the fish and the plant samples (root and shoot) also has been determined after the drying step.

Sediment samples from the replicates could only be collected at the last three weeks of the trial, as none was available in the initial stage. Sediment samples collected weekly from week three directly from the mechanical filter throw the outlet valve then sent to the ALEC. On the last day of the experiment, the sediment samples collected after squeezing the filter media by hand into each reservoir. At the ALEC, to extract the metals from the sediment, the samples were centrifuged at 3000 rpm for 15 min then to dry the samples, water disposed from the tubes using a plastic pipette and finally, before the digestion step, tubes were subjected to air dried by loose the caps of the tubes. All intermediate and final

sample weights were recorded. Good Laboratory Practices were followed for all handling, labeling, and recording of sample data.

All samples were stored after collection and processing in a clean and sterilized cooler with ice packs and promptly (less than 6 hours) transported to the ALEC lab for HMs determination.

In the lab, and before analyzed the samples for HMs, fish, plants, fish feed, and the sediments samples subjected for digesting step at the ALEC lab.

Collecting

Weekly ten ml water samples were collected in 15 ml plastic tubes from day 1 (week 1) until the last week of the experiment (week 6). Fish feed samples were collected before starting the experiment and stored in 50 ml sterile plastic tubes. The ground fish samples were stored in 50 ml sterile plastic tubes. Plant samples after grinding of the edible portion and for the root were stored in 50 ml sterile plastic tubes. Weekly sediment samples were collected (starting from week 3) and stored in a sterile 50 ml plastic tubes.

Laboratory analysis

All samples were sent to the ALEC lab for analyzing. HMs were determined after extraction from the samples using inductively–coupled plasma mass spectrometry (ICP-MS).

Water quality tests

Each replicate's water sample had several water quality parameters including ammonia, nitrate, dissolved oxygen, water temperature, and electrical conductivity determined.

Metals analysis by ALEC lab

Tissue sample preparation and digestion:

Well-ground tissue samples (< 100um) were oven-dried at 60°C and stored in a desiccator. The microwave-assisted acid digestion procedure (modified from US EPA Method 3051) is a closed vessel technique which uses 0.1 to 0.5g of sample material plus 1mL concentrated nitric acid (Omni-trace HNO_3, EMD Chemicals), (1mL hydrogen peroxide (30%, Suprapure, EMD) if requested) and 1mL ultrapure water (18Ω). Digestion was performed in MARS6 microwave digestion system (CEM Corp., Matthews, North Carolina).

Analysis by Inductively Coupled Plasma Mass Spectrometry (ICCP-MS)

Solutions were analyzed on an Agilent Model 7700x ICP-MS (Santa Clara, CA). Instrument parameters used are listed in the list of tables (Table. 29.

Prior to initial calibration, a daily performance check serves to verify instrument response over the mass range from Lithium to Thallium, to monitor background noise level and the presence of oxides (CeO/Ce) and doubly-charged ions (Ba++/Ba) which must be less than three percent.

Calibration standards were prepared from multi-element stock solutions (except for Hg, which is a single element standard) purchased from AccuStandard (New Haven, CT).

The stocks were diluted in 1% nitric acid to provide a working calibration curve of at least 5 points. Samples were also diluted with 1% nitric acid until their response is determined to be within the calibration range. Internal standards (Rh,

In and Ga) are added to both standards and samples prior to analysis using a mixing tee in the sample introduction system.

Quality Control measures

Following the US EPA protocol in Method 6020, each run includes quality control checks referred to as Initial Calibration Verification (ICV) standards and Independent Calibration Verification. These QC checks must fall within +/- 10% of their expected value.

A mid-range standard was analyzed after every 10 samples and again at the end of the run. These QC checks are referred to as Continuing Calibration Verification (CCV) samples, and the results must fall within 25% of the expected value.

In addition, a QC solution sample (such as NIST 1643e Trace metals in water) was chosen to match the matrix of the samples to be analyzed and is included at the beginning and end of each sample set.

Statistical analysis

For analyzing the data, values of P used to compare for statistical significance ($P < 0.05$) of different means were determined between the treatment and the control using t-test for equal n. Six weeks of water data, data for three weeks of the sediments (week 3 to week 6) were analyzed while the data of the fish and the lettuce (root and shoot) were analyzed by comparing the first day of the experiment with the last day.

Results

Parameters

During the study period, environment parameters were in a normal range for the air temperature, the relative humidity, and the photosynthetic active radiation were 22.2 C°, 44.81%, and 43.4 μmole/m^2/s, respectively from Jan 8th (day 1) to Feb 12th (day 35) of 2018. Also, the range of the pH was 8.2-7.0, the DO was 6.5-5.8 mg/l, the EC from 0.4-0.7 mS/cm f, the Tw were 23.6-26 C°, the ammonia (NH_3) was 0.05-0.25 mg/l, and the average of nitrate (NO_3) was 3.1-22.2 mg/l.

Heavy metals in the water

Arsenic

As expected, As level increased gradually during the trial in both the control and the treatment. In the control probably as a function of concentration due to evapotranspiration and continued introduction of fish feed. Arsenic level increased in the spiked treatment significantly more ($P < 0.05$) (Fig 1). Although, as general concentration, final arsenic levels were within the MCL (9.31 per 10 μg/L) of the drinking water standard (EPA, 2018); the levels in two of the treatment replicates had accumulated close to the limit (Table. 2) while one replicate (Treatment 2) exceeded the MCL.

Figure. 1. Average arsenic concentration of the control and the treatment in the water during the experiment period with the MCL for the arsenic (EPA, 2018). The error bars represent the standard deviation (S.d).

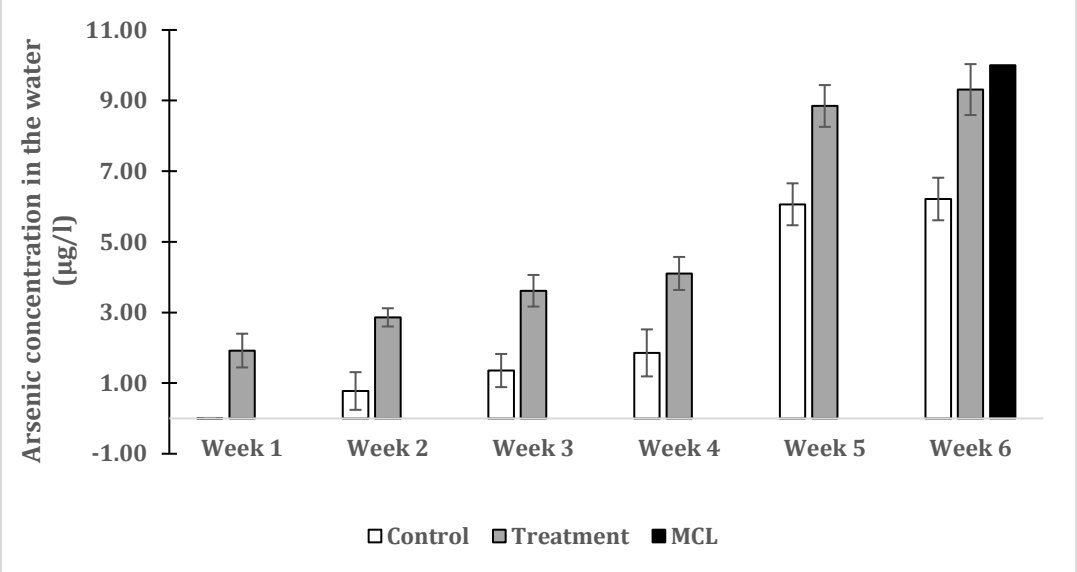


Table 2. Arsenic concentration (µg/l) of the control and the treatment in the water during the experiment period. P-value (means of the control and the treatment). MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Week1	Week2	Week3	Week4	Week5	Week6	P-value
Control 1	0.00	0.49	1.14	1.86	5.62	6.05	0.016
Control 2	0.02	1.52	2.01	2.67	6.90	7.02	
Control 3	0.00	0.31	0.92	1.04	5.66	5.57	
Treatment 1	1.80	2.62	2.99	3.47	8.17	8.34	
Treatment 2	2.56	3.22	4.02	4.59	9.61	10.06	
Treatment 3	1.40	2.75	3.83	4.25	8.76	9.54	
MCL				10			µg/L

Cadmium

Cd did not accumulate in the water (Fig 2), and its concentration were within the MCL in the water (2.1 per 5 µg/L) (EPA, 2018). Cd concentrations in the water fluctuated for both the control and the treatment over the time. Their levels decreased by week 4 then increased again at the last week of the trial by the end

of the trial, but there was no difference between the treatment concentrations with the controls ($p > 0.05$) (Table. 3).

Figure. 2. Average cadmium concentration of the control and the treatment in the water during the experiment period with the MCL for the arsenic (EPA, 2018). The error bars represent the standard deviation (S.d).

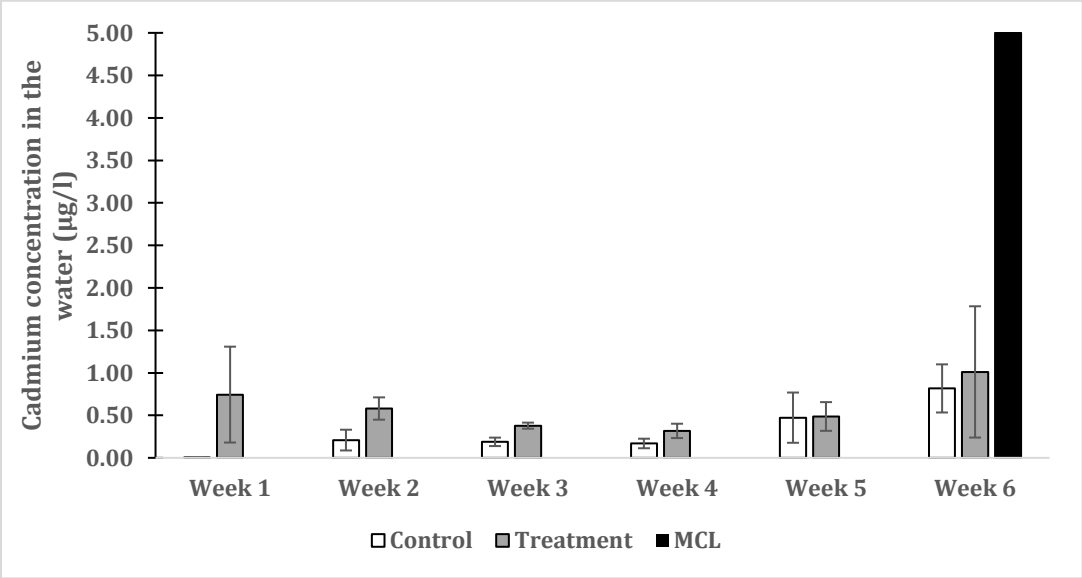


Table 3. Cadmium concentration (µg/l) of the control and the treatment in the water during the experiment period. P-value (means of the control and the treatment). MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	P-value
Control 1	0.01	0.11	0.22	0.14	0.86	0.50	0.052
Control 2	0.00	0.13	0.12	0.12	0.15	1.18	
Control 3	0.00	0.38	0.23	0.25	0.40	0.77	
Treatment 1	0.20	0.40	0.36	0.21	0.25	2.10	
Treatment 2	0.51	0.72	0.43	0.42	0.55	0.42	
Treatment 3	1.52	0.62	0.35	0.33	0.65	0.51	
MCL				5			µg/L

Mercury

By the last week of the experiment, Hg concentration of the treatment decreased to a very low levels well below the MCL (0.007 per 2 µg/L) of the

drinking water standard (EPA, 2018) (Fig 3), and there was a significant difference comparing to the control ($P < 0.05$) (Table. 4).

Figure. 3. Average mercury concentration of the control and the treatment in the water during the experiment period with the MCL for the arsenic (EPA, 2018). The error bars represent the standard deviation (S.d).

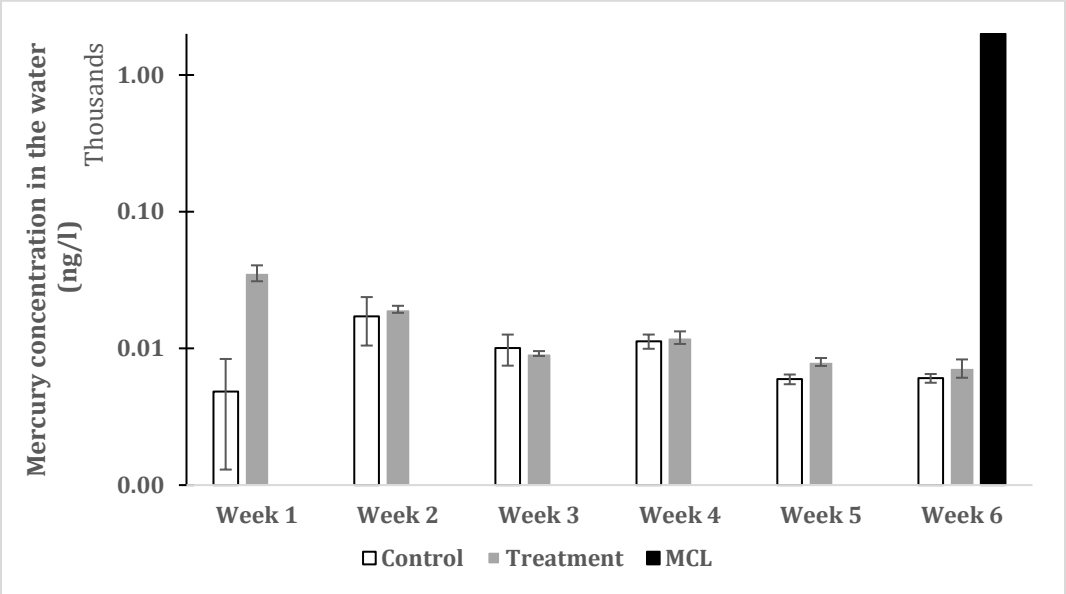


Table 4. Mercury concentration (ng/l) of the control and the treatment in the water during the experiment period. P-value (means of the control and the treatment). MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Week1	Week2	Week3	Week4	Week5	Week6	P-value
Control 1	0.40	9.65	13.15	11.75	5.37	6.61	0.041
Control 2	9.05	25.75	6.85	9.45	5.94	6.02	
Control 3	5.05	15.95	10.15	12.65	6.56	5.52	
Treatment 1	30.35	19.35	9.45	11.15	7.43	7.22	
Treatment 2	34.95	20.75	9.45	13.85	8.70	8.55	
Treatment 3	41.95	17.95	8.65	11.15	7.79	5.85	
MCL	2000						ng/L

Lead

Pb was not accumulated in the water over the trial time. The average concentration for both the control and the treatment decreased to low levels, so

they were within the MCL (0.05 per 15 µg/L) of the drinking water standard (EPA, 2018) (Fig 4). There was no difference between the treatment and the control ($p > 0.05$) (Table. 5).

Figure. 4. Average lead concentration of the control and the treatment in the water during the experiment period with the MCL for the arsenic (EPA, 2018). The error bars represent the standard deviation (S.d).

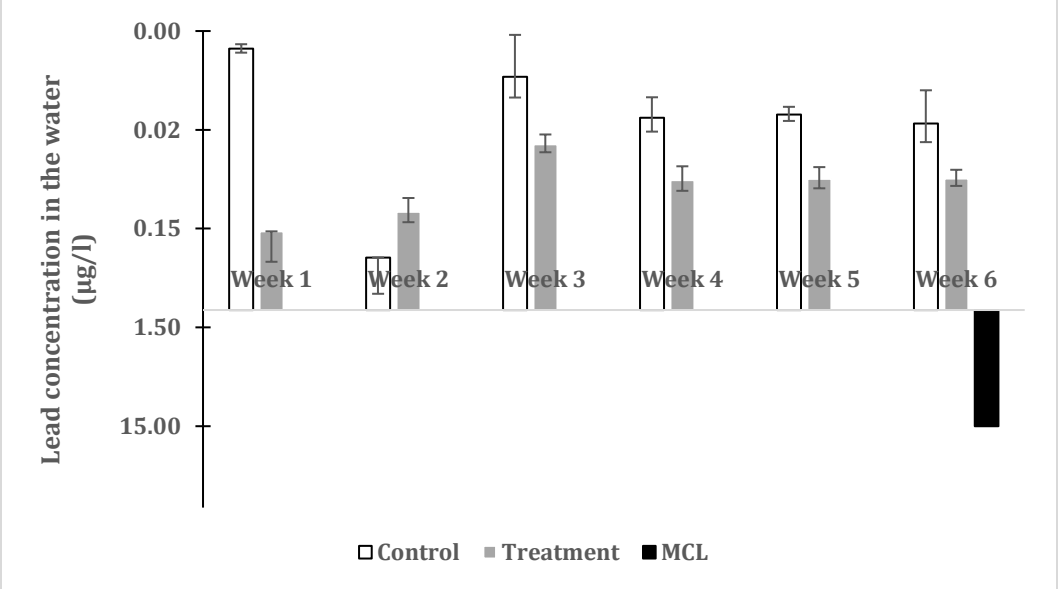


Table 5. Lead concentration (µg/l) of the control and the treatment in the water during the experiment period. P-value (means of the control and the treatment). MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	P-value
Control 1	0.00	0.01	0.01	0.02	0.01	0.01	0.771
Control 2	0.00	0.03	0.00	0.01	0.01	0.02	
Control 3	0.00	0.85	0.00	0.01	0.01	0.01	
Treatment 1	0.03	0.07	0.02	0.03	0.03	0.04	
Treatment 2	0.05	0.14	0.03	0.07	0.06	0.04	
Treatment 3	0.39	0.09	0.02	0.05	0.05	0.06	
MCL			15				µg/L

Heavy metals in the fish tissue

Arsenic

Compared to the background levels of arsenic in fish tissue for the control and the treatment in the first day of the trial, arsenic did not accumulated in the treatment by the end of the trial. There was no difference between the treatment and the control of arsenic concentration in the fish tissue ($P > 0.05$) (Fig 5) and (Table. 6).

Figure. 5. The average of arsenic concentration (dry weight) of fish tissue of control and the treatment at the first and last day of the experiment duration. The error bars represent the standard deviation (S.d).

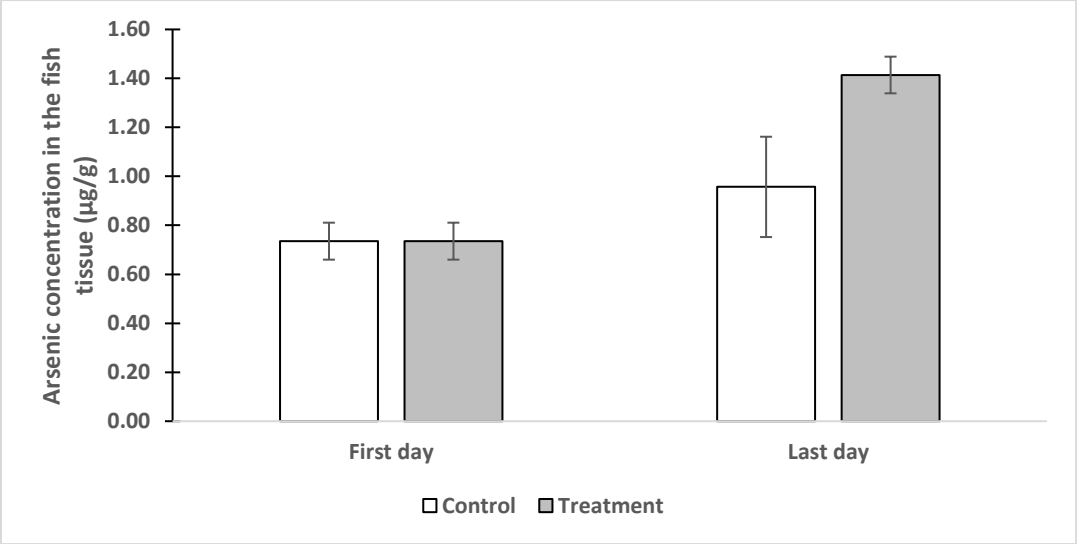


Table 6. Total arsenic concentration (µg/g) of the control and the treatment (dry weight) in the fish tissue for the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.74	0.74	0.074
Control 2	0.74	0.89	
Control 3	0.74	1.23	
Treatment 1	0.74	1.41	
Treatment 2	0.74	1.33	
Treatment 3	0.74	1.51	

Cadmium

Both the control and the treatment of the cadmium concentration decreased at the end of the study compared with the initial concentration at day 1, so they did not accumulate in the fish tissue, and there was no difference between the treatment and the control ($P > 0.05$) (Fig 6) and (Table. 7).

Figure. 6. The average of cadmium concentration (dry weight) of fish tissue of control and the treatment at the first and last day of the experiment duration. The error bars represent the standard deviation (S.d).

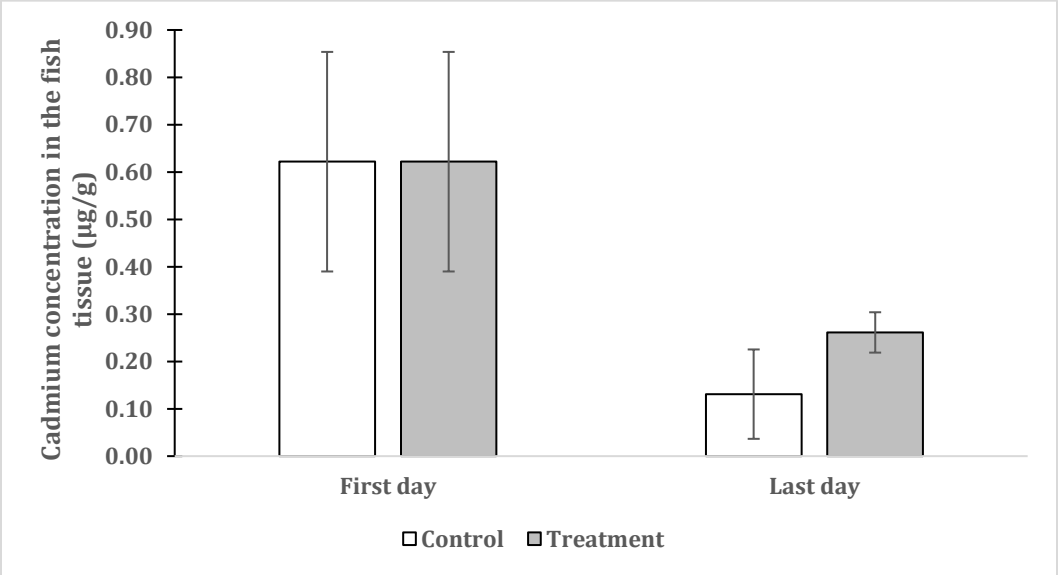


Table 7. Total cadmium concentration (µg/g) of the control and the treatment (dry weight) in the fish tissue for the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.62	0.06	0.18
Control 2	0.62	0.07	
Control 3	0.62	0.26	
Treatment 1	0.62	0.27	
Treatment 2	0.62	0.31	
Treatment 3	0.62	0.21	

Mercury

Based on the wet weight, Hg accumulated in the fish tissue of the treatment by the end of the trial (Fig. 7) and (Table 8). Comparing to the control, the Hg (methylmercury) level of the treatment (29.86 ng/g) increased significantly by the last day of the study ($P < 0.05$). However, it was within the maximum allowable level (MAL) for methylmercury in fresh fish (500 ng/g) set by the joint FAO/WHO expert committee on food additives (FAO/WHO, 1995). The total Hg concentration was determined as wet weight after conversion from the dry weight. However, the standard for the Hg provided in the MAL was for the methylmercury, but not for the total Hg which we analyzed.

Figure. 7. The average of mercury concentration (ng/g) of the control and the treatment in the fish tissue (wet weight) for the last day of the experiment with the MAL by FAO/WHO standard (FAO/WHO, 1995). The error bars represent the standard deviation (S.d).

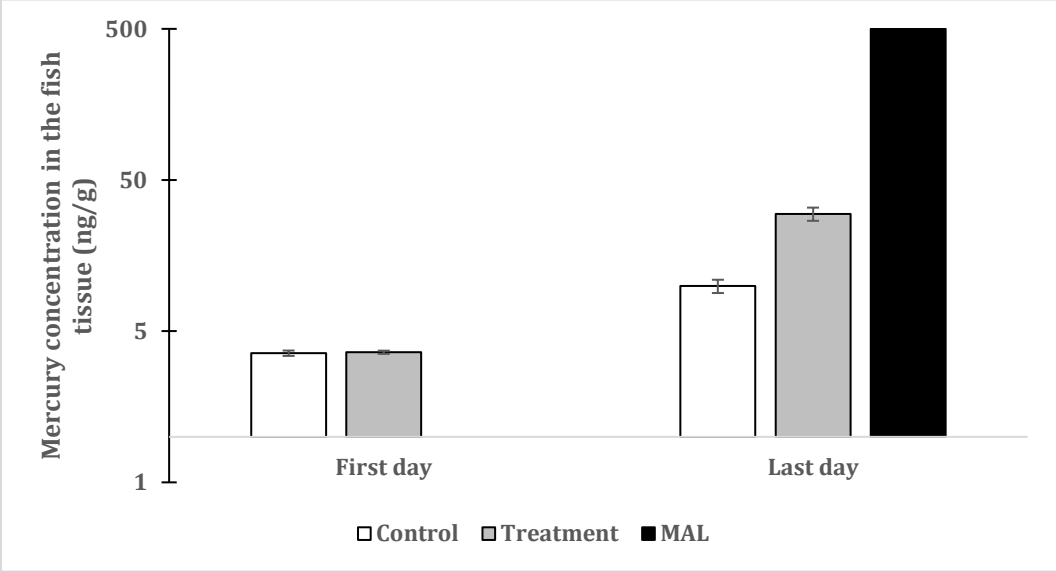


Table 8. Total mercury concentration (ng/g) of the control and the treatment in the fish tissue (wet weight) for the first and the last day of the experiment. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard.

	First Day	Last Day	P-value
Control 1	2.59	8.63	0.006
Control 2	2.66	11.05	
Control 3	2.43	10.16	
Treatment 1	2.52	27.91	
Treatment 2	2.67	34.08	
Treatment 3	2.62	27.59	
MAL	500		ng/g

Lead

Based on the wet weight, Pb accumulated in the fish tissue by the end of the trial (Fig. 8) and (Table 9); the Hg level of the treatment increased significantly comparing to the control ($P < 0.05$). However, the Pb level was within the MAL of fresh fish (104.1 per 300 ng/g) that set by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 1995).

Figure. 8. The averages of lead concentration (wet weight) of fish tissue for the control and the treatment at the first and last day of the experiment duration with the MAL by FAO/WHO standard (FAO/WHO, 1995). The error bars represent the standard deviation (S.d).

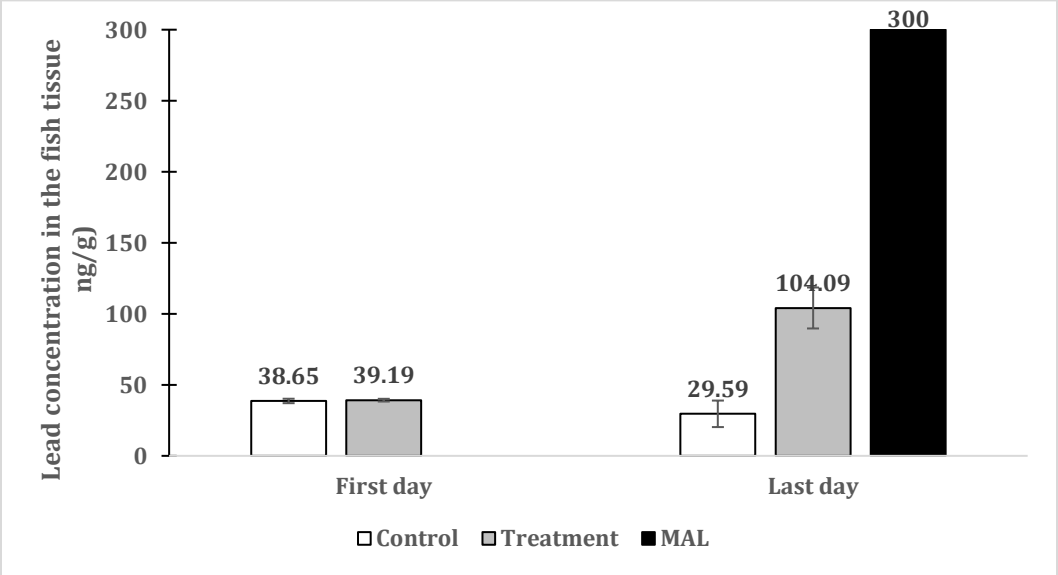


Table 9. Total lead concentration (ng/g) of the control and the treatment in the fish tissue (wet weight) for the first and the last day of the experiment. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard.

	First Day	Last Day	P-value
Control 1	38.90	21.42	
Control 2	40.43	24.70	
Control 3	36.61	42.65	0.006
Treatment 1	37.93	108.44	
Treatment 2	40.26	84.66	
Treatment 3	39.38	119.18	
MAL	300		ng/g

Heavy metals in the lettuce tissues

Arsenic

Arsenic did not accumulate in the lettuce (shoot tissue) by the end of the trial and there was no difference between the treatment and the control ($P > 0.05$) (Fig 9) (Table. 10).

Figure. 9. The averages of arsenic concentration (dry weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

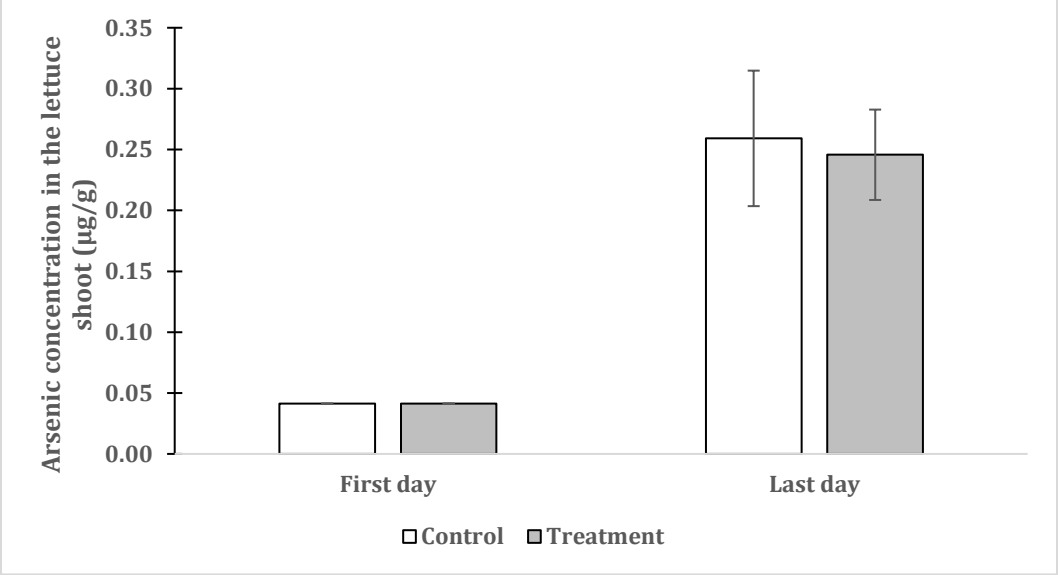


Table 10. Arsenic concentration (dry weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.04	0.20	0.792
Control 2	0.04	0.25	
Control 3	0.04	0.33	
Treatment 1	0.04	0.30	
Treatment 2	0.04	0.23	
Treatment 3	0.04	0.21	

Arsenic accumulated in the root of the lettuce tissue by the end of the trial; there was a significant difference between the control and the treatment ($P < 0.05$) (Fig 10), and (Table. 11).

Figure. 10. The average of arsenic concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

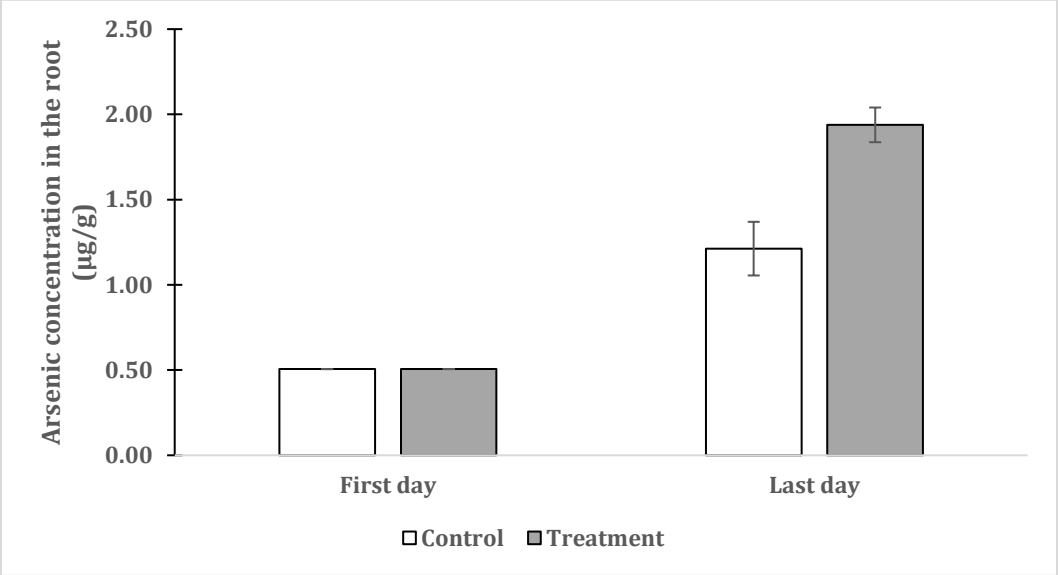


Table 11. Arsenic concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.51	1.06	0.008
Control 2	0.51	1.15	
Control 3	0.51	1.43	
Treatment 1	0.51	1.83	
Treatment 2	0.51	2.07	
Treatment 3	0.51	1.92	

Cadmium

Based on the wet weight, Cd did not accumulate in the lettuce (shoot tissue) of the treatment by the end of the trial. The Cd levels were within the MAL of leafy vegetables (0.12 per 0.2 µg/g) set by FAO/WHO, and there was no difference between the treatment and the control ($P > 0.05$) (Fig 11) (Table. 12).

Figure. 11. The average of cadmium concentration (wet weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment with the MAL by FAO/WHO standard (FAO/WHO, 1995). The error bars represent the standard deviation (S.d).

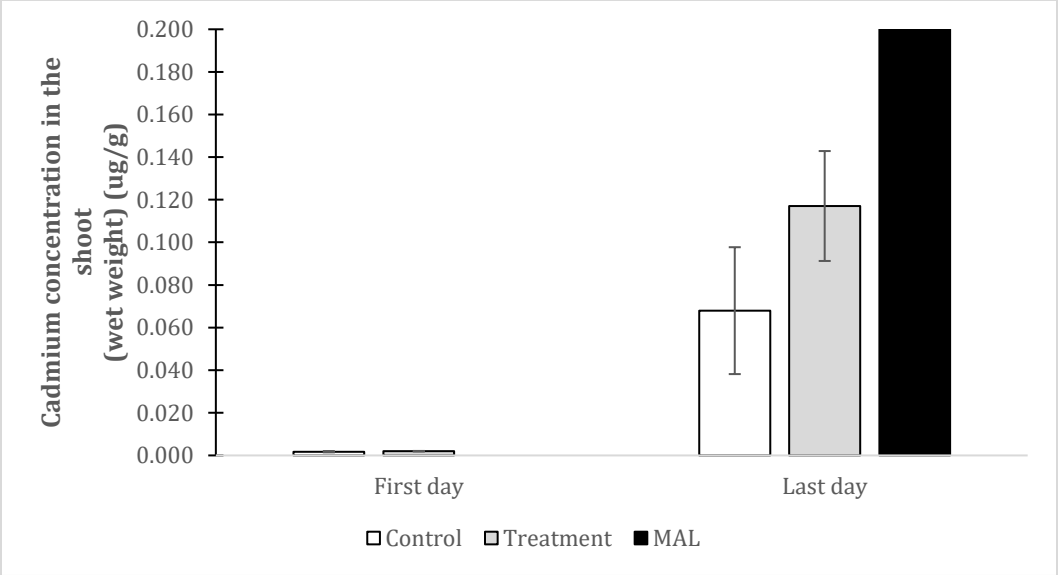


Table 12. Cadmium concentration (wet weight) (ng/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard (FAO/WHO, 1995).

	First Day	Last Day	P-value
Control 1	1.9	29.5	0.154
Control 2	2.0	72.2	
Control 3	1.3	102.1	
Treatment 1	1.9	91.8	
Treatment 2	2.1	152.5	
Treatment 3	1.8	107.0	
MAL	200		ng/g

By the end of the study, Cd concentrations had not accumulated in the root of the lettuce tissue in the treatment; there was no difference between the cadmium levels in the control and the treatment ($P > 0.05$), (Fig 12) and (Table. 13).

Figure. 12. The average of cadmium concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

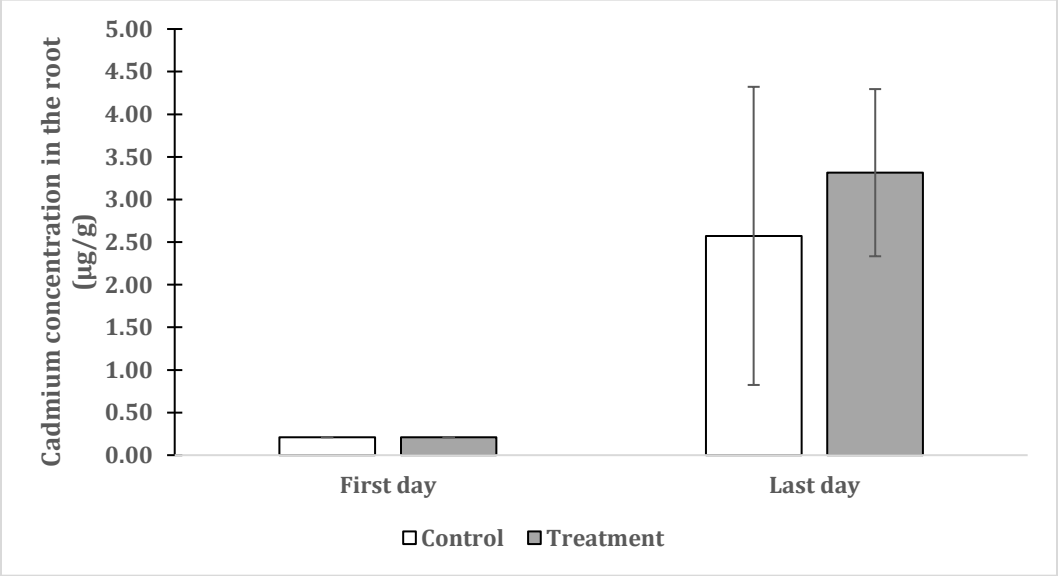


Table 13. The average cadmium concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.21	0.92	0.636
Control 2	0.21	1.81	
Control 3	0.21	4.99	
Treatment 1	0.21	2.30	
Treatment 2	0.21	3.00	
Treatment 3	0.21	4.64	

Mercury

By the end of the experiment, mercury had not accumulated in the shoot of the lettuce tissue in the treatment: there was no difference comparing with the control ($P > 0.05$) (Fig 13) (Table. 14).

Figure. 13. The average of mercury concentration (dry weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

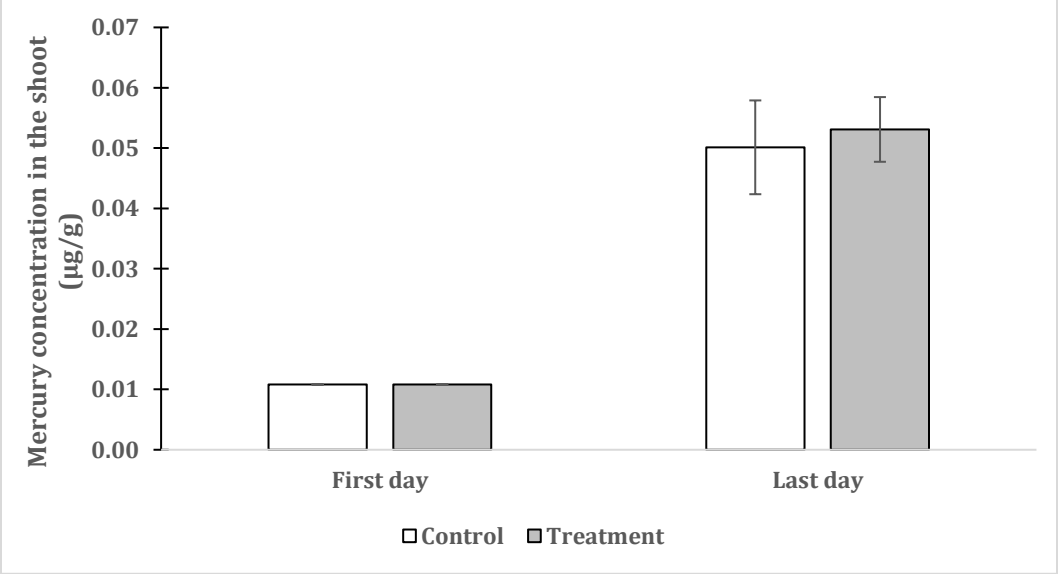


Table 14. Mercury concentration (dry weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.01	0.05	0.683
Control 2	0.01	0.04	
Control 3	0.01	0.06	
Treatment 1	0.01	0.06	
Treatment 2	0.01	0.05	
Treatment 3	0.01	0.05	

Hg accumulated in the root of the treatment, and there was a difference compared to the control ($P < 0.05$) (Fig 14), and (Table. 15).

Figure. 14. The average of mercury concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

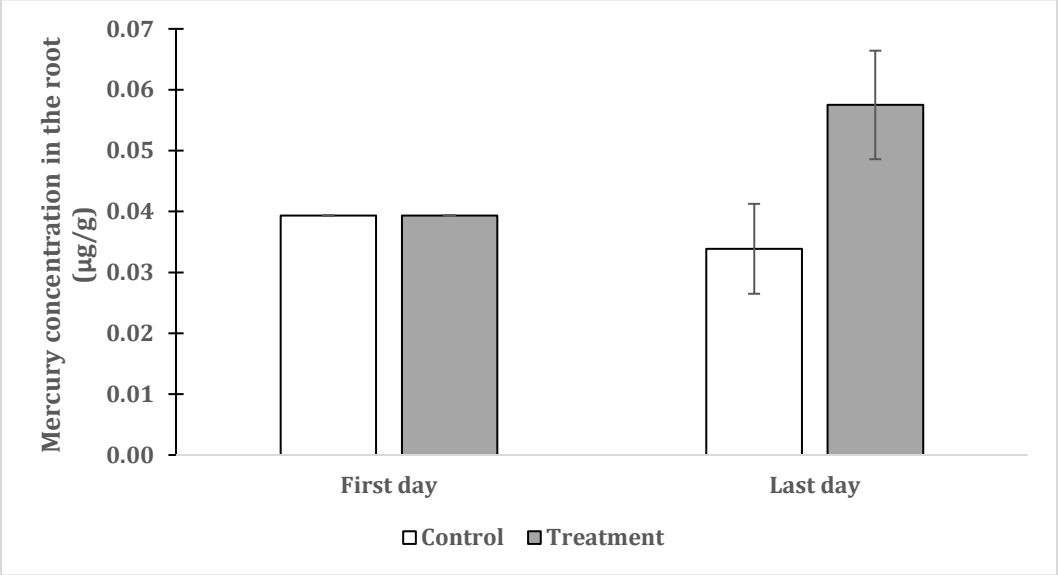


Table 15. Mercury concentration (dry weight) (µg/g) of the control and the treatment in the lettuce root for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	0.04	0.03	0.047
Control 2	0.04	0.03	
Control 3	0.04	0.04	
Treatment 1	0.04	0.05	
Treatment 2	0.04	0.06	
Treatment 3	0.04	0.07	

Lead

Based on the wet weight, Pb had not accumulated in the shoot of the lettuce (Fig. 15), and there was no difference compared to the control (P >0.05) (Table. 16). The concentration of the Pb were way below the MAL for leafy vegetables (0.003 per 0.3 µg/g) set by FAO/WHO standard (FAO/WHO, 1995).

Figure. 15. The averages of lead concentration (wet weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment with the MAL by FAO/WHO standard (FAO/WHO, 1995). The error bars represent the standard deviation (S.d).

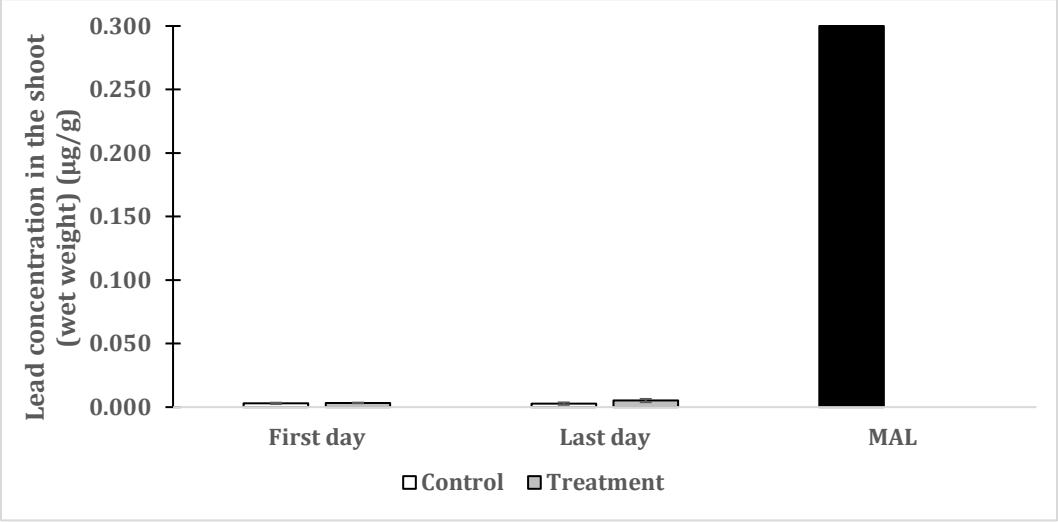


Table 16. Lead concentration (wet weight) (µg/g) of the control and the treatment in the lettuce shoot for the first and the last day of the experiment. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard (FAO/WHO, 1995).

	First Day	Last Day	P-value
Control 1	0.003	0.004	0.099
Control 2	0.003	0.003	
Control 3	0.002	0.002	
Treatment 1	0.003	0.006	
Treatment 2	0.004	0.006	
Treatment 3	0.003	0.003	
MAL	0.3		µg/g

Pb concentrations accumulated in the root of the lettuce tissue of the treatment, and there was a significant difference between the Pb levels in the control and the treatment ($P < 0.05$) (Fig 16), and (Table. 17).

Figure. 16. The average of lead concentration (dry weight) of the control and the treatment in the root of the lettuce tissue for the first and the last day of the experiment. The error bars represent the standard deviation (S.d).

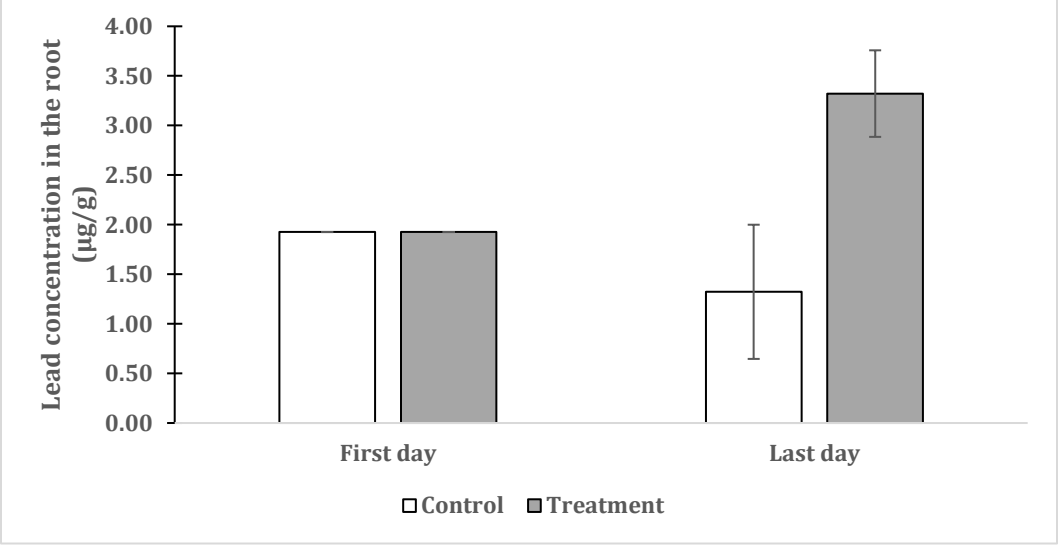


Table 17. Lead concentration (dry weight) (µg/g) of the control and the treatment in the root of the lettuce tissue for the first and the last day of the experiment. P-value (means of the control and the treatment).

	First Day	Last Day	P-value
Control 1	1.93	2.28	0.032
Control 2	1.93	0.90	
Control 3	1.93	0.79	
Treatment 1	1.93	2.91	
Treatment 2	1.93	3.13	
Treatment 3	1.93	3.93	

Heavy metals in the sediment

Arsenic

Unexpectedly, As level decreased after the week 4 (the second week of sediment sampling) for the control and the treatment as well, and there was no difference between them by the end of the trial ($P > 0.05$) (Fig 17) (Table. 18).

Figure. 17. The average of arsenic concentration of the control and the treatment in the sediment during the experiment period. The error bars represent the standard deviation (S.d).

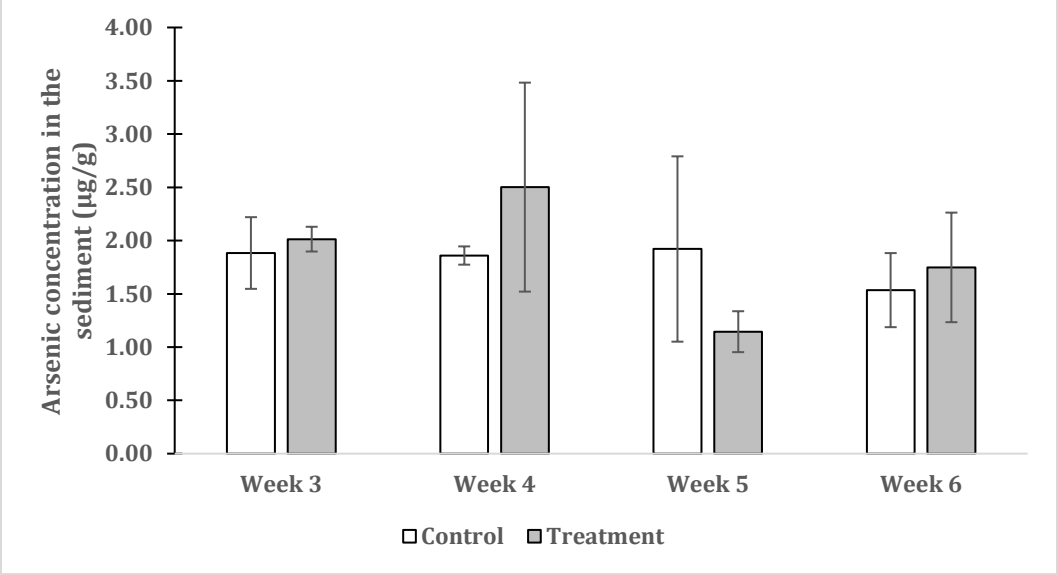


Table 18. Total arsenic concentration (µg/g) of the control and the treatment in the sediment during the experiment period. P-value (means of the control and the treatment).

	Week3	Week4	Week5	Week6	P-value
Control 1	2.35	1.95	3.08	1.46	0.741
Control 2	1.71	1.88	1.71	1.99	
Control 3	1.58	1.75	0.98	1.15	
Treatment 1	2.02	1.45	1.10	1.13	
Treatment 2	1.87	3.81	0.93	1.72	
Treatment 3	2.15	2.24	1.40	2.39	

Cadmium

Although Cd levels tended to be higher for both the control and the treatment by the fourth week (the second-week sampling of sediments), they decreased later in week 5 and week 6, the end of the experiment. Cd did not accumulate also in the sediment, and there was no difference comparing to the control by the end of the trial ($P > 0.05$) (Fig 18) (Table. 19).

Figure. 18. The average of cadmium concentration of the control and the treatment in the sediment during the experiment period. The error bars represent the standard deviation (S.d).

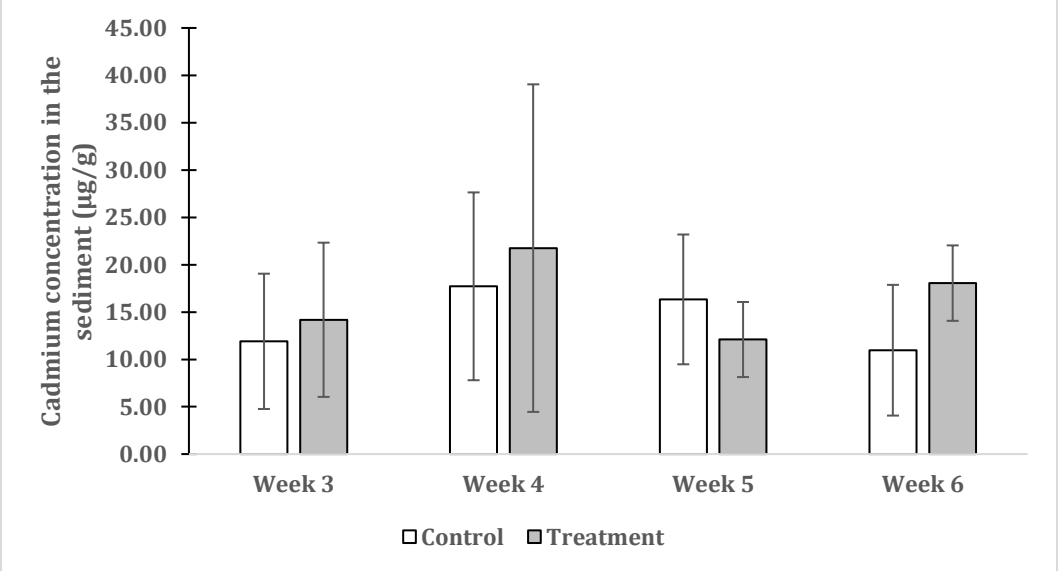


Table 19. Total cadmium concentration (µg/g) of the control and the treatment in the sediment during the experiment period. P-value (means of the control and the treatment).

	Week3	Week4	Week5	Week6	P-value
Control 1	10.89	30.41	16.25	4.06	0.579
Control 2	3.71	6.20	7.99	20.40	
Control 3	21.12	16.55	24.78	8.45	
Treatment 1	4.73	6.50	7.62	15.86	
Treatment 2	24.61	45.94	17.26	23.65	
Treatment 3	13.22	12.81	11.42	14.66	

Mercury

Hg behaved in a similar manner to As and Cd. It did not accumulate in the treatment, and there was no difference between the treatment and control by the end of the trial (P>0.05) (Fig 19) (Table. 20).

Figure. 19. The average of mercury concentration of the control and the treatment in the sediment during the experiment period. The error bars represent the standard deviation (S.d).

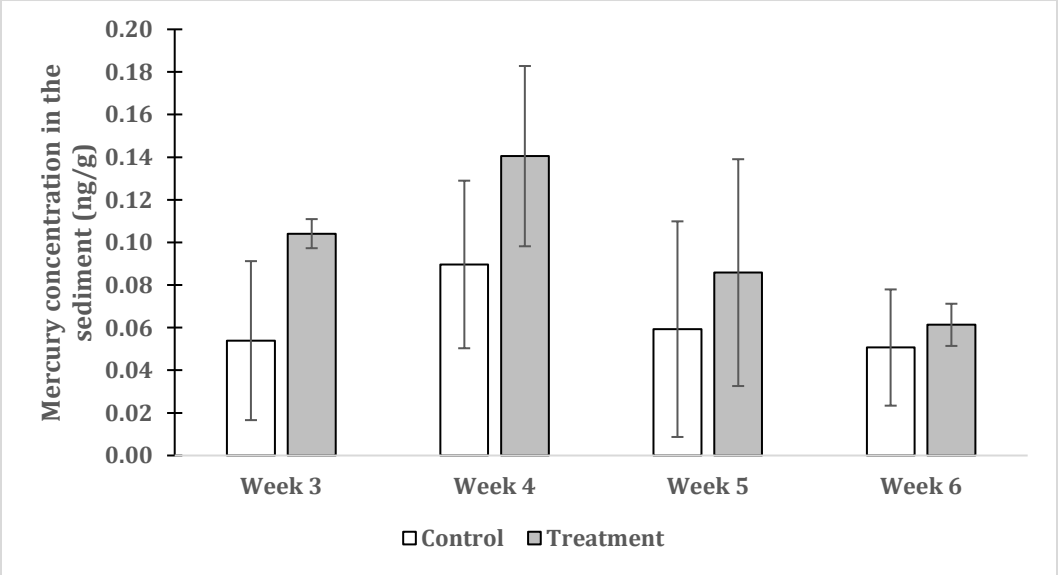


Table 20. Total mercury concentration (µg/g) of the control and the treatment in the sediment during the experiment period. P-value (means of the control and the treatment).

	Week3	Week4	Week5	Week6	P-value
Control 1	0.107	0.073	0.129	0.087	0.077
Control 2	0.027	0.052	0.012	0.022	
Control 3	0.029	0.144	0.037	0.043	
Treatment 1	0.095	0.147	0.161	0.050	
Treatment 2	0.106	0.189	0.047	0.060	
Treatment 3	0.111	0.086	0.049	0.074	

Lead

Lead accumulated significantly by the end of the trial ($P < 0.05$) compared to the control (Fig 20) (Table. 21).

Figure. 20. The average of lead concentration of the control and the treatment in the sediment during the experiment period. The error bars represent the standard deviation (S.d).

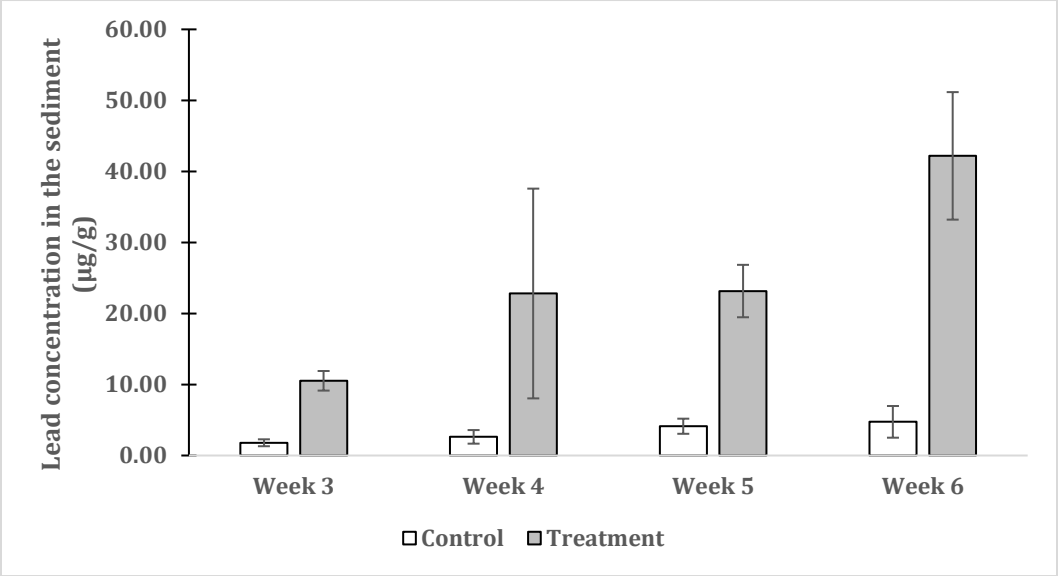


Table 21. Total lead concentration (µg/g) of the control and the treatment in the sediment during the experiment period. P-value (means of the control and the treatment).

	Week3	Week4	Week5	Week6	P-value
Control 1	2.27	2.09	4.42	3.02	0.0004
Control 2	1.99	1.81	2.69	3.32	
Control 3	1.13	3.97	5.25	7.89	
Treatment 1	9.78	11.80	25.75	49.06	
Treatment 2	12.46	43.68	17.94	29.51	
Treatment 3	9.33	12.94	25.78	47.99	

Finally, the HMs distribution among the system components (water, fish, shoot, root, and sediment) and in the bio-balls (Table. 22) and (Fig. 21; 22; 23; 24; 25; 26; 27; 28) showed the trend of the concentration of the HMs to be distributed in the system.

Table. 22. The concentration distribution of the HMs in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

	Water (µg/L)	Fish (µg/g)*	Shoot (µg/g)*	Plant root (µg/g)*	Sediment (µg/g)	Bio-balls (µg/L)
As	9.312	1.414	0.246	1.938	1.748	5.960
Cd	1.011	0.261	2.733	3.314	18.056	36.038
Hg	0.007	0.114	0.053	0.058	0.061	89.300
Pb	0.047	0.401	0.120	3.322	42.188	263.609

*Dry weight sample

Figure. 21. The concentration distribution of As in the treatment components (water, fish, shoot, root, sediment, and the bio-balls) over the study period (35 days)

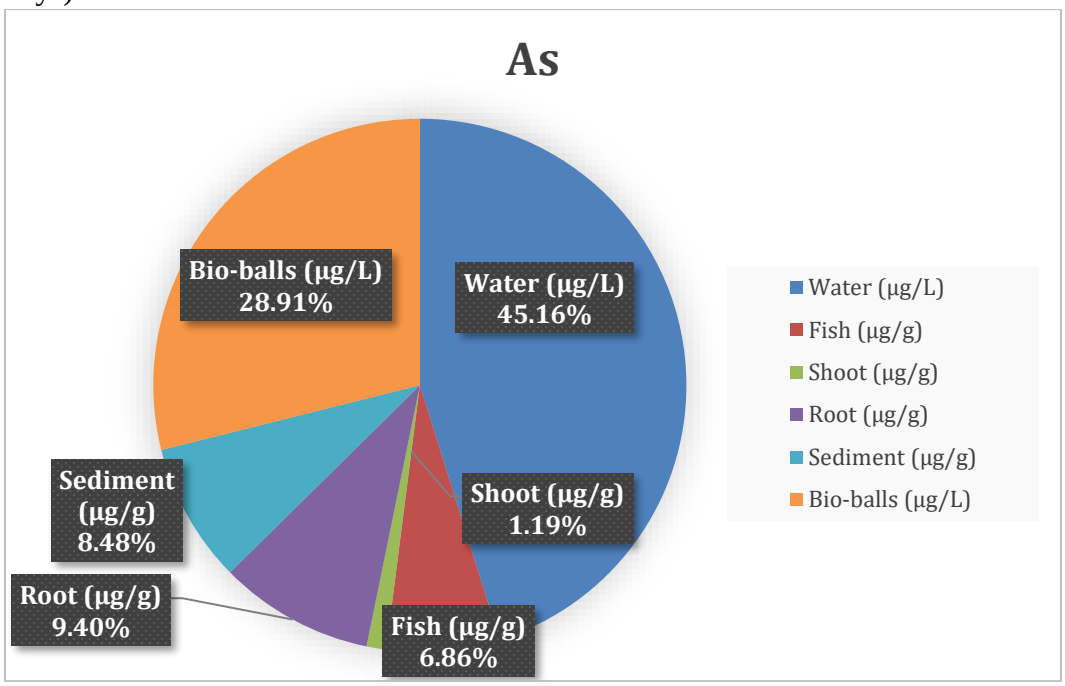


Figure. 22. The concentration distribution of Cd in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

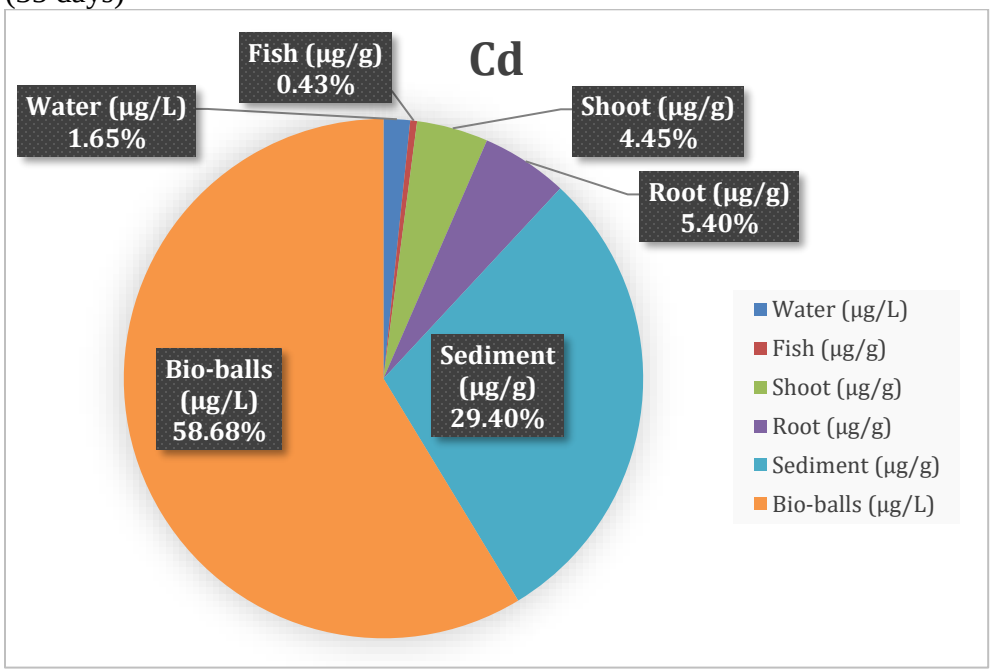


Figure. 23. The concentration distribution of Hg in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

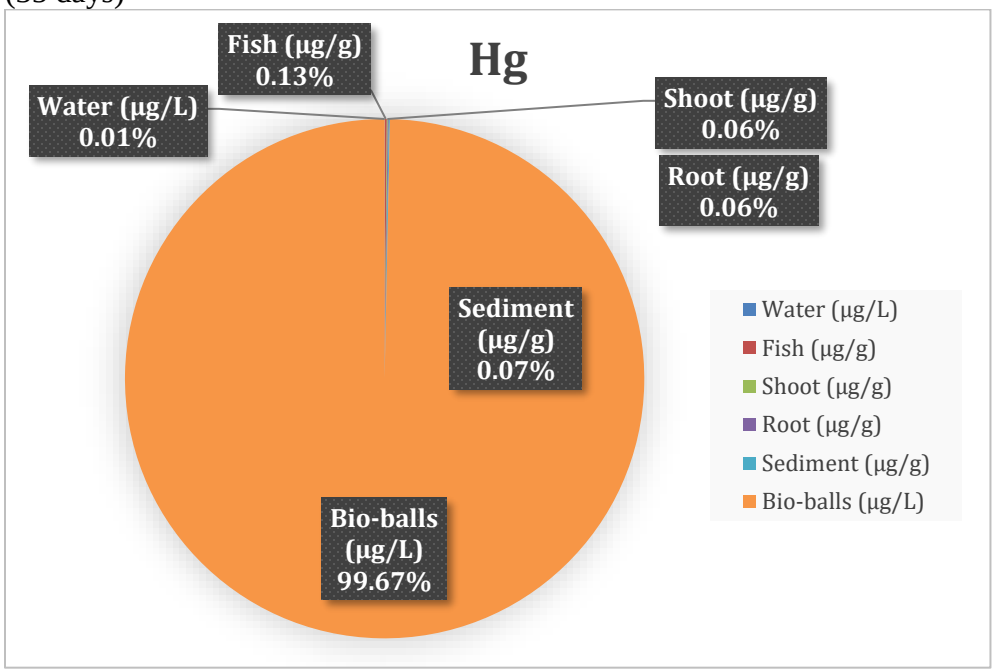


Figure. 24. The concentration distribution of Pb in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

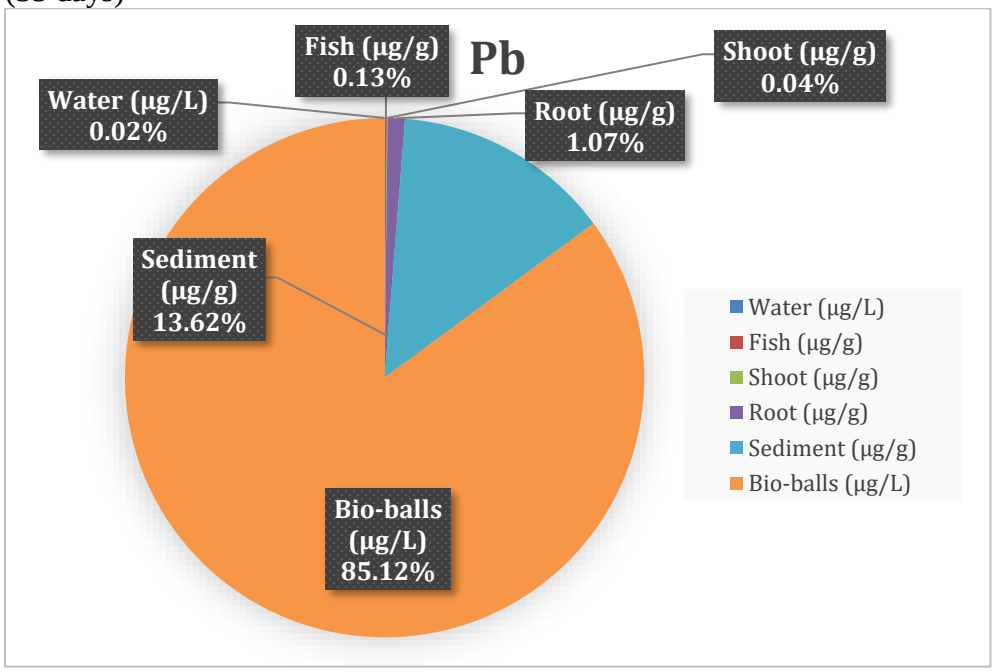


Figure. 25. The concentration distribution of the HMs in the treatment components (water, fish, shoot, root, and sediment which include the of the HMs in the bio-balls) over the study period (35 days)

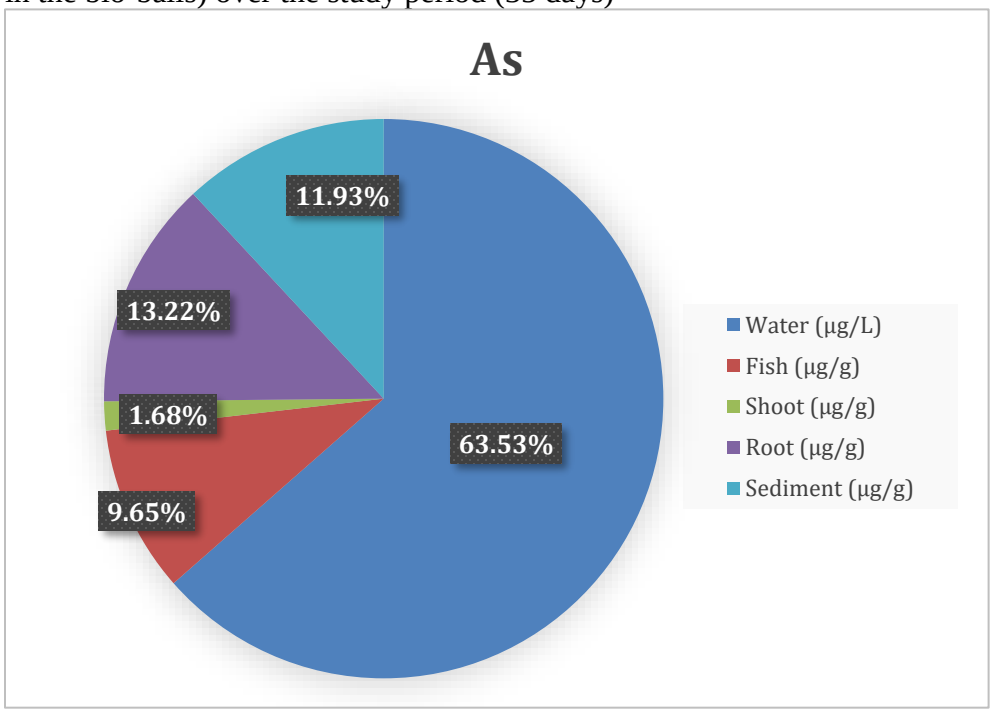


Figure. 26. The concentration distribution of the HMs in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

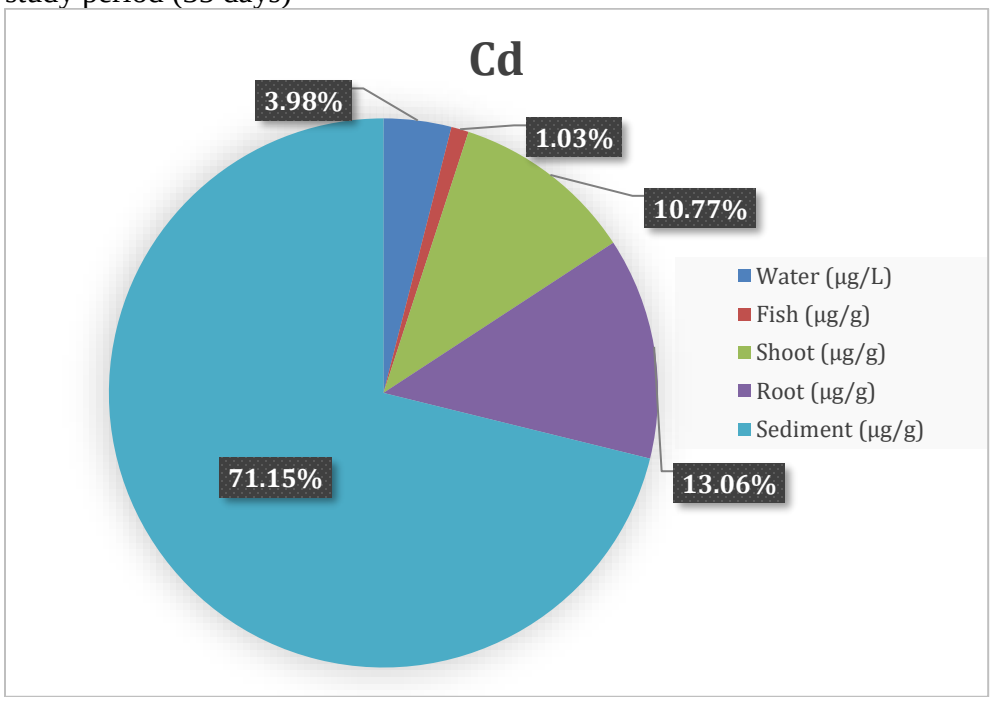


Figure. 27. The concentration distribution of the HMs in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)

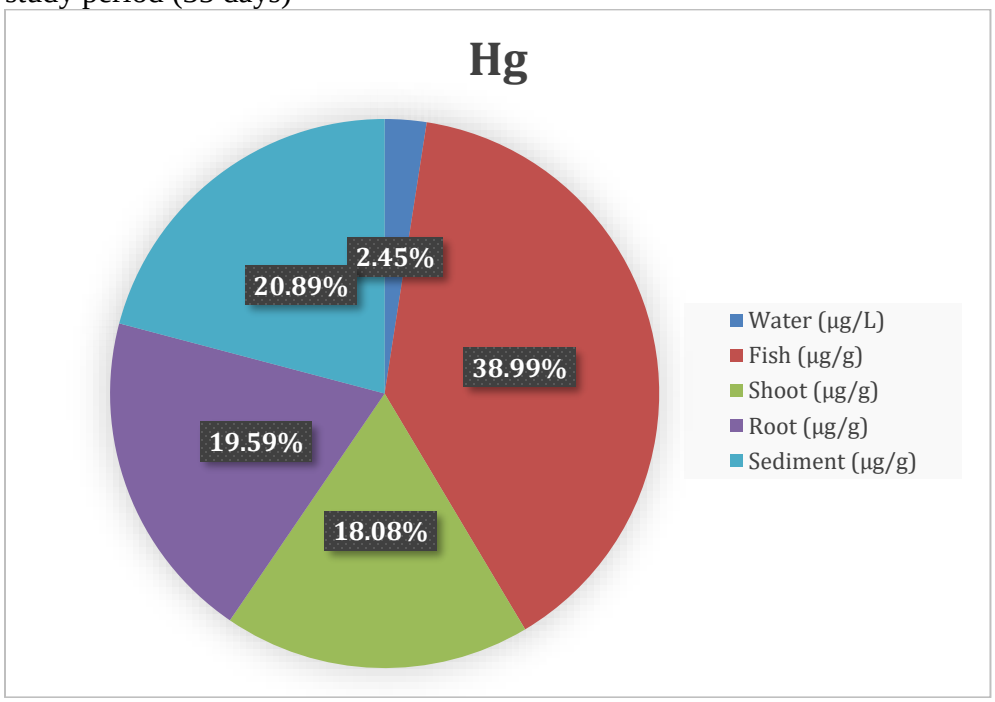
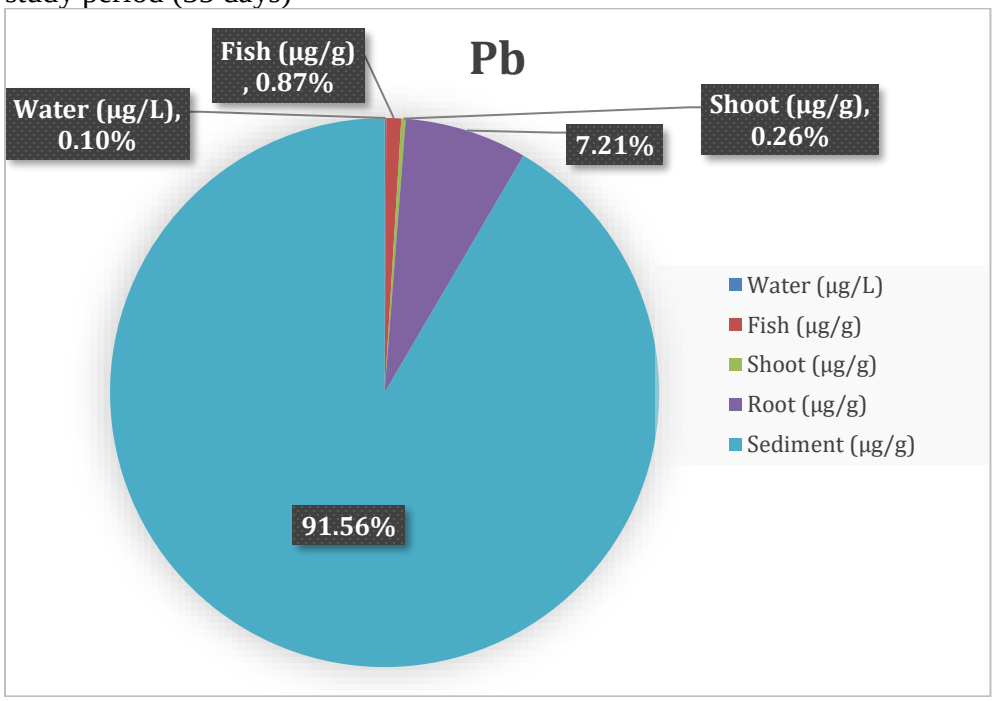


Figure. 28. The concentration distribution of the HMs in the treatment components (water, fish, shoot, root, and sediment) and in the bio-balls over the study period (35 days)



Discussion

Operators and regulators of aquaponic systems are interested in the mechanisms and fates of heavy metals accumulation, fractionation, and distribution behavior in the water, the fish, the plant shoot, the plant root, and the sediment. We will review and discuss how each metal behaved in the water, in the fish, and the edible portion and the root portion of the plants. Finally, the sediments will be discussed as one group. With regards to the metals tested, the US Environmental Protection Agency has published MCL in drinking water while the world organizations like FAO and WHO have established additional limits for foods. We follow the EPA MCL of the drinking water on each metal, but there are only two MAL for the methylmercury and the Pb in fish tissue. As mentioned above, microbes in the water are known to produce methylmercury from anthropogenic mercury pollution. So, increases in the environmental concentrations of Hg may increase methylmercury as well (CDC, 1999). The average proportion of methylmercury bioaccumulation increase from the aqueous environment has been reported for phytoplankton, zooplankton, and fish to be 5%, 15%, 30%, and more than 90%, respectively (Crump and Trudeau, 2009; Watras and Bloom, 1992). Therefore, it may be most of the total Hg was in a methylmercury form. However, we need to analyze the methylmercury in the samples to provide an accurate concentration. In addition to Hg and Pb in fish samples, the two published AML's for Cd and Pb in edible plants also will be included in the discussion.

There were no comparable previous studies on aquaponics available to compare the results with this study. However, there are a few studies focused on the heavy metals in aquaculture or hydroponic systems. Other studies reviewed the potential accumulation of heavy metals in soil. However, none were directly focused on the accumulation of metals in one integrated unit like aquaponics system.

As the results showed in the current study, comparing to the control, As accumulated significantly in the water by the end of the experiment ($P < 0.05$) (Fig. 1) (Table. 2). It was expected As increased in the water over the time due to two reasons. First, the fish feed has higher concentrations of As compared to the other metals that studied in this study (Fig. 30) (about 1.38 µg/g comparing to 0.05, 0.02, and 0.33 for Cd, Hg, and Pb, respectively). Second, as water was evaporated from the system, As was left behind in the system water. Although As in the treatment did not exceed the MCL, as average concentration of all replicates, its concentration reached to relatively high levels during the trial period when spiked with only a 20% of MCL (7.96µg/L; 0.11 µM) of the arsenic compound (Na_2HAsO_4). Although, as general concentration, final As levels reached to high level compared to the MCL (9.31 per 10 µg/L) of the drinking water standard (EPA, 2018); the levels in two of the treatment replicates had accumulated close to the limit (Table. 2) while one replicate (Treatment 2) exceeded the MCL. Seasonality could have an effect on the As and other heavy metals concentration in the system. For example, As may have exceeded the MCL

due to the summer's effect on the water evaporation from the tank and the reservoir, and also from the plant because of the transpiration process. In addition, MCL could be exceeded if the initial water supply or the fish feed had included higher concentrations. The concentration of As in the fish feed can be higher based on its source. For instance, As was reported in fish feed products to limit levels close to being excluded from the market (Schenone et al., 2014).

Figure. 29. The arsenic background concentration in tap water compared to the MCL based on the EPA standard (EPA, 2018).

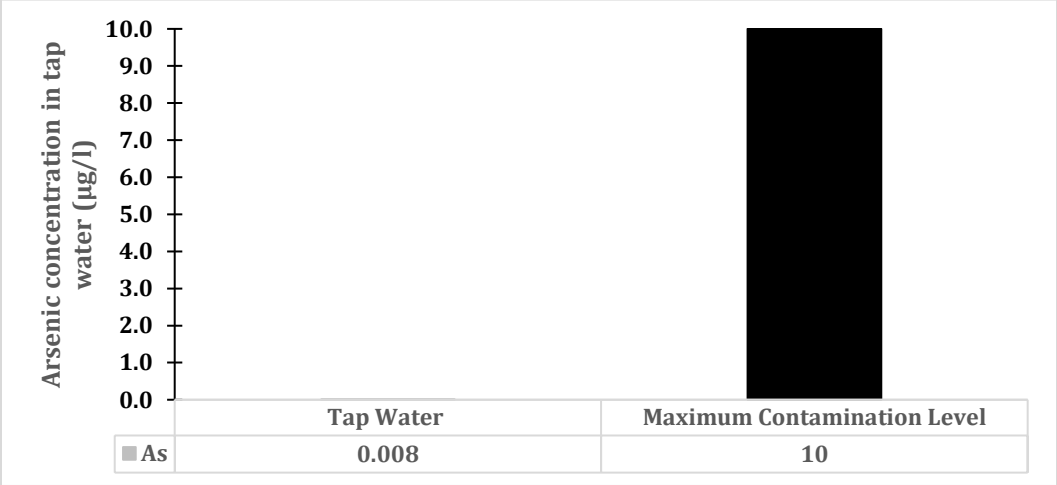
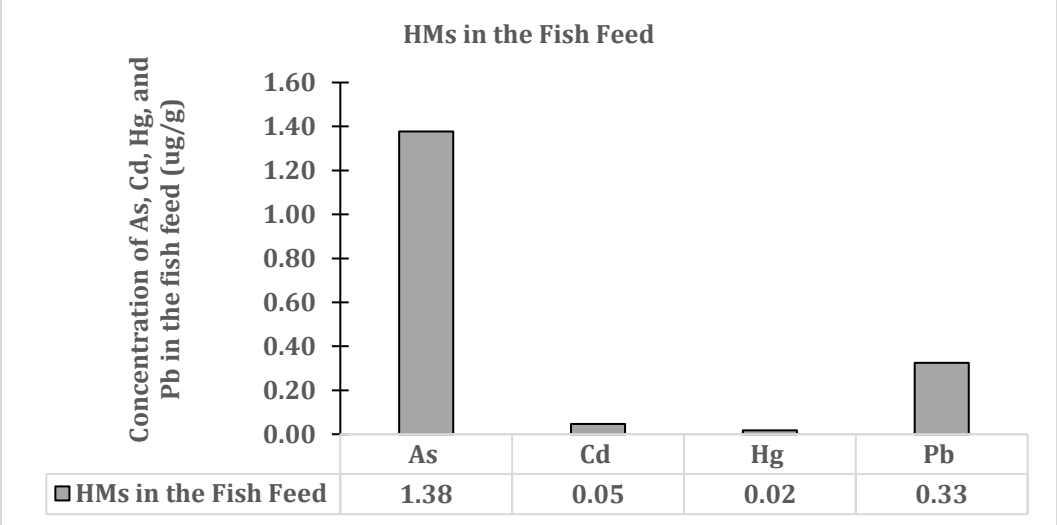


Figure. 30. The concentration of the studied heavy metals (arsenic, cadmium, mercury, and lead) in the fish feed (µg/g).



Heavy metals uptake into the fish tissue commonly depends on their concentration in the water (Schenone et al., 2014). Ohki and Maeda. (2002) studied bioaccumulation and biotransformation of arsenic compounds in tilapia (*O. mossambicus*) after exposure of the fish to un-dosed and As-dosed diets. They found that the accumulation of As in tilapia was proportional to the As concentration in the feed. Also, approximately 90% of the arsenic accumulated by the fish was depurated to water, after the fish were transferred into As-free water for a day. In the present study, although the As was in a high level (9.31 µg/l) in the water by the end of the trial, it did not accumulate in the fish tissue (1.41 µg/g dry weight) ($P > 0.05$) (Fig. 5) and (Table. 6). This may indicate that high level of As may not be a concern with the fish tissue over the same period studied, however, As may bioaccumulate if the fish rearing in the system for a longer period.

Martins et al. (2011) studied the effect of different RAS systems with different daily water exchange rates (30, 70 and 1500 l/kg feed/d) on the concentration of some heavy metals (As, Cd, Pb, and others) in the water of the culture system and tissues of Nile tilapia (*Oreochromis niloticus*). The results showed that some metals concentrations such as As, Fe, Mn, Ni, and Zn increased in the water as water exchange rates decreased. The authors suggested that heavy metals accumulated in the system with the higher rate of water recirculated. After 71 days of the experiment period, As concentrated in the culture water when water exchange decreased and concentrated in the fish tissue. In our experiment, As

levels also increased by the last day of the trial in the water of the treatment replicates, but the statistical analysis showed the metal did not accumulate in the fish (P=0.074) compared to the control. However, in Martins study, the system ran for more than two months with more fish cultured in the system which presumably means more feed and nutrients were provided, thus more metals could be available to the fish. In comparison, our study used fewer fish and shorter experimental period (about one month) which may impact the As bioaccumulation in the fish even if the As concentration increased in the water.

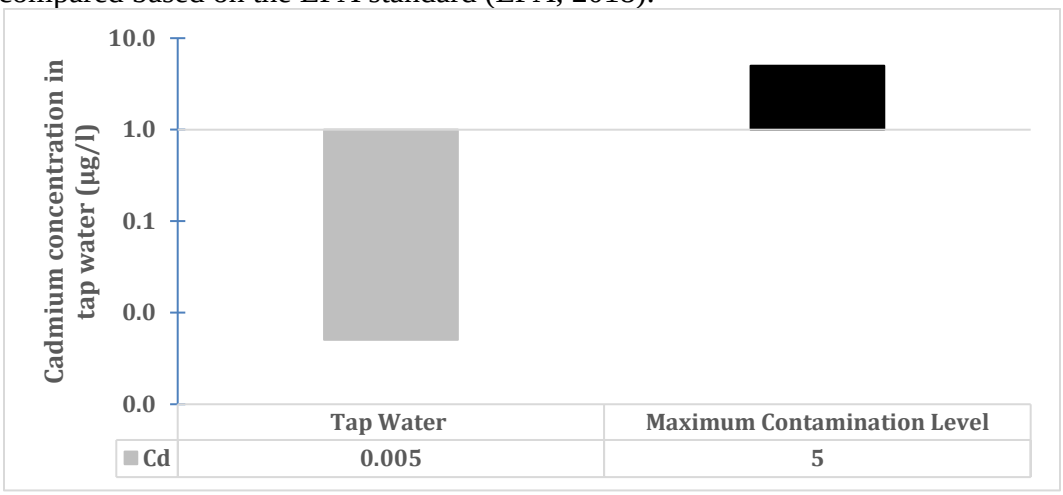
Associated with the high concentration of As in the water of the treatment, it accumulated in the plant root tissue ($P < 0.05$) (Table. 11), but did not in the shoot ($P > 0.05$) (Table. 10). As may not have bioaccumulated in the lettuce shoot due to the ability of the root of the plant to sequester As within the root area. There is a mechanism which may explain why the plant avoids As accumulation in the shoot portion. A physical defense mechanism of the lettuce, functions through plant root border cells, releasing from the root cap of plants to confront potential hazards or contaminants like heavy metals in the surrounding environment (Tran et al., 2016; Tollefson et al., 2015). As produced in many plants, root tip extracellular matrix include border cell populations (Huskey et al., 2018). The root border cells are attached structures that cover plant roots tips like a sheath. The plant roots were possibly defending by trapping metals which can then be immobilized (Huskey et al., 2018; Hawes et al., 2016; Tran et al., 2016; Tollefson et al., 2015; Curlango-Rivera et al., 2014; Driouich et al., 2013).

Cobb et al. (2000) studied some metals (Pb, Cd, and Zn) including As in different plant tissues including iceburg lettuce (*Lactuca sativa*) to determine uptake and distribution of the metals tested in the edible part and the root of the plants when grown in mine wastes and in soils mixed with mine wastes in five different treatments (0, 25, 50, 75, and 100% mine tailing). As effectively accumulated and translocated in lettuce roots and leaves with similar concentrations; no statistical difference was found of the As concentrations in lettuce parts. The author suggested that the low of nutrient and organic matter content in the soil were factors that cause higher accumulation of the metal, more than expected. However, the concentrations and the media used is different than what we have in the current study. Besides, the defense mechanism mentioned about the border cells in the root area of the plant could be playing a role to bioaccumulate the As in the root. It could be indicated that the plant was able to tolerate As concentration at the current levels, but they may be able to tolerate the higher levels reported by Cobb et al. (2000).

In the present study, Cd concentration in the water of the treatment was tended to be higher compared to the control. However, based on the statistical results of the Cd in the water ($P < 0.05$) (table. 3), Cd did not accumulate by the end of the experiment when spiked with 15% of the MCL=15 µg/L (1.76 µg/L; 0.02 µM) of (EPA, 2018). Distribution of the Cd in the fish tissue, plant tissue (shoot and root), and the sediment may have an effect on its accumulation in the water. Also, its low concentration in the background water (0.005 µg/L) comparing to its MCL (5 µg/L) (Fig. 31) and in the fish feed (0.05 µg/g) (Fig. 30) which is the primary source for

Cd in the system, effected on its accumulation which reflected on the accumulation and bioaccumulation in the other samples as well (as will discuss later).

Figure. 31. The cadmium background in the water and the MCL in the tap water compared based on the EPA standard (EPA, 2018).



The analysis of Cd in the fish tissue showed no tendency to accumulate in the treatment, and there was no difference comparing with the control ($P > 0.05$) (Fig 6) and (Table. 7). Cd did not accumulate in the water over the experiment period (35 days) which was reflected in low levels in the fish tissue (as dry weight). Martins et al. (2011) found that Cd did not accumulate in the liver and the muscle of fish (Nile tilapia (*Oreochromis niloticus*)) over time. They found that although Cd concentration in the water increased when they decreased the water exchange rate, it did not bioaccumulate in the fish for all treatments (rates of water exchange). Similarly, in our results, Cd did not accumulate. Also, the bioavailability of the metals may be minimized by the physicochemical properties of the water (Martins et al., 2011).

According to Le Croizier et al. (2018), it is necessary to study the bioaccumulation of heavy metals over several months because metals can take several months to bioaccumulate in the fish tissue. Also, the small fish we used could not accumulate Cd like the older fish used in the previous study; larger fish can bioaccumulate more Cd concentrations throughout their life than younger fish do (El-Moselhy et al., 2014). According to several field studies of heavy metals accumulation in fish tissues, Cd and Hg are accumulated at very low levels, below 1 µg/g dry weight (Jezierska and Witeska, 2006). This concurred with the current study results; Cd concentrations in the treatment did not exceed 0.31 µg/g dry weight of the fish tissue in the replicates of the treatment (Fig. 6) (Table. 7).

For the plant samples, Cd concentration converted to the wet weight to compare the Cd concentration of the shoot sample with the standard of FAO/WHO (Fig. 11) (Table. 12). The Cd concentration the edible part (the shoot) was within the MAL of leafy vegetables (0.117 per 0.2 µg/g), and there was no difference between the treatment and the control ($P > 0.05$). Also, although the Cd tended to be higher in the root of the treatment than in the control by the last day (Fig. 12), the statistical analysis showed there was no difference with the control (Table. 13) which means there was no accumulation also in the root of the lettuce. It may Cd concentration tested in the study was lower than the limit that can be accumulated in the shoot or the root of the lettuce. Cobb et al. (2000) determine the concentrations of Cd and other metals (Pb, As, and Zn) in different plants including iceberg lettuce. The plants cultured in mine wastes and in soils mixed

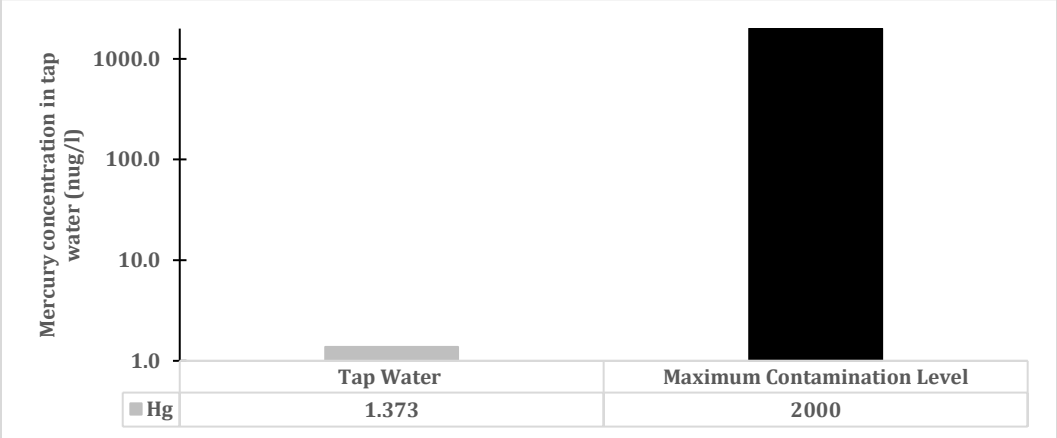
with mine wastes in five different treatments (0, 25, 50, 75, and 100% mine tailing), and in general, Cd accumulated in similar concentrations in the roots and the leaves of the lettuce as dry weigh; lettuce roots of the treatment grown in 100% mine waste was significantly higher in Cd concentrations compared to the control. Also, Cd was not immobilized in the lettuce roots, and it accumulated more in the leaves. This result may show how the high concentration in the water can have an effect on the bioaccumulation of the Cd in the plant tissues. However, genetic characteristics also can play a significant role. Zorrig et al. (2019) evaluated mechanisms controlling of Cd tolerance and accumulation in different lettuce that represent large genetic diversity. They suggested this statement "root Cd concentration and root-shoot Cd translocation were under a complex genetic determinism involving at least two loci." and there is a possible limit for both the accumulation of Cd in root or shoot and translocation of the metal from the shoot to the root. However, this will also depend on recessive loci.

Ratios of Cd:Zn can play an important role in the accumulation of Cd in the plants. Zare et al. (2018), found Zn concentration in hydroponic nutrient solution play an essential role for reducing level of Cd in the inner cells of the root (root symplastic), and decrease Cd and translocation into the xylem as well as significantly reduced Cd transport to and accumulation in the top part of the plant. Therefore, determine Cd:Zn ratio may provide more details that help to understand the additional factors that may affect the distribution and accumulation of Cd in the lettuce tissue.

Hg concentration of the treatment decreased to very low levels of the MCL

(0.007 per 2 µg/L) compared to the first week (0.036 per 2 µg/L) of the drinking water standard (EPA, 2018) (Fig 3), and there was a significant difference comparing to the control ($P < 0.05$) (Table. 4). However, the metal fractions accumulated in the fish tissue by the end of the experiment. In addition to the reflection of the Hg from the water to the fish tissue, it can evaporate naturally from the water body. According to the Penman. (1948), Hg can naturally evaporate from open water. Dissolved metallic Hg evaporates at faster rates than the ionic or adsorbed mercury forms (Mackay and Wolkoff, 1973); this can significantly influence its persistence in bodies of water which may explain the decreasing level in the treatment and control water.

Figure. 32. The mercury background concentration in tap water compared to the MCL based on the EPA standard (EPA, 2018).



Hg concentration in the fish feed was the lowest concentration comparing to the other metals (0.02 µg/g of fish feed) (Fig. 30). Also, we have tested a very low level of the metal (1.5%) of the MCL=2 µg/L (0.04 µg/L; 4 x 10^{-5} µM) as methylmercury chloride (CH_3HgCl) in the water of the treatment systems.

However, the analysis of Hg based on the wet weight showed highly tending to accumulate in the fish tissue (Fig. 7). Although the increased level of the metal was significant ($P < 0.5$) (Table. 8), its concentration in the fish tissue was within the MAL (29.9 per 500 ng/g wet weight) (FAO/WHO, 1995) (Fig. 7) and (Table. 8). However, this standard for the Hg as methylmercury, but not the total Hg which what we tested.

In addition to the availability of the Hg in the water, increasing concentration of Hg in the fish tissue depends on several factors such as speciation of Hg, methylation and de-methylation rates, a concentration of dissolved organic carbon. The effect among these factors may be linked to each other (Grieb et al., 1990). Methylmercury is the primary form of Hg found in freshwater and marine fish (Rolfhus and Fitzgerald, 1995), and it can concentrate and be bioaccumulated in fish tissue (CDC, 1999). According to the study done by Grieb et al. (1990), to determine the relationship between physicochemical characteristics of lakes located in U.S. and Hg concentrations in fish tissue showed 99% of the mercury in fish muscle tissue was in the (CH_3Hg^+) form. So converting the metallic form of Hg forms in water between inorganic mercury (Hg^{2+}) and organic form (CH_3Hg^+) mostly depends on methylation process of bacteria (sulfate reducers) (Kidd and Batchelar, 2011). Also, Crump and Trudeau. (2009) stated that CH_3Hg^+ can bioaccumulate in fish mainly by the uptake of food, and its concentration in the tissue affected by several factors such as fish age, and species. According to Spry and Wiener (1991), fish can accumulate higher concentrations of Hg, Cd, and Pb in

the acidic water (pH≤6.5), and the increasing bioaccumulation of these metals may refer to their aqueous form abundances (Cd^{2+}, CH_3Hg^+, and Pb^{2+}) at lower pH levels. The pH levels decreased in our study from 8.2 to 7.0. The author and his collogues also mentioned that the decrease of the calcium (Ca) in the water increase the CH_3Hg^+ and divalent metal ions permeability to the membranes of the fish-gill due to the inverse relationship between them because of the competing with metals ions that binding the same sites on the gill surface. The fish food that often used in aquaponics farms are not supplied of Ca at required quantities due to the absorption of the Ca in fish and plant tissue as well. Fish fed over the trial period compensated the loss of any potential loss of the Hg concentrations due to the potential evaporation from the water over the time. Hall et al. (1997) conducted a field experiment to determine the degree of (CH_3Hg^+) accumulated in finescale dace fish (*Phoxinus neogaeus*) via their food or through passive uptake from water through the gills. The fish were held in 2000 L enclosed pens floating in an oligotrophic lake in Ontario, Canada. Fish exposed to different levels of methylmercury in the water either at low (0.10–0.40 ng/L, intermediate (0.45–1.30 ng/L), or at high (0.80–2.1 ng/L) concentrations. The fish were fed zooplankton with different levels of methylmercury; low (0.16–0.18 µg/g dry weight), or high (0.28–0.76 µg/g dry weight). At natural levels of methylmercury, they found that food is the dominant pathway of the methylmercury bioaccumulation in fish tissue. However, we may need to analyze the methylmercury in the future studies instead of the total Hg in order to provide accurate data for the human health field.

Several field studies of heavy metals accumulation in fish tissues reported that mercury accumulates at very low levels, below 1 µg/g dry weight (Jezierska and Witeska, 2006). In our study, total Hg was between (0.01-0.11 µg/g dry weight) in the treatment replicates. The concentration range of Hg we have in the current study through the trial duration as wet weight was less than the study of Chahid et al. (2014); they evaluated some heavy metals concentrations including Hg in different species of fish from various fishing ports of the Kingdom of Morocco. They found the range of Hg in the samples analyzed was (0.05–0.194 µg/g wet weight) compared to (0.02-0.03 µg/g wet weight) in the current study. The results showed that Hg was within the maximum levels set by the EU for the fish and shellfish (0.5 µg/g wet weight) ("Commission Regulation," 2006).

The root tissue (dry weight) accumulated Hg, and there was a significantly difference compared with the control ($P < 0.05$) (Fig. 14) and (Table. 15). The roots of the treatment were able to sequester greater concentrations than the initial levels of the Hg of the first day (48-69 per 39 ng/g). However, although Hg increased in the shoot tissue by the end of the trial (Fig. 13) compared to the result of the first day, the statistical analysis showed there was no difference compared to the control ($P> 0.05$) (Table. 14).

According to Shariatpanahi and Anderson (1986), different vegetables were cultured in several contaminated sites that accumulated different heavy metals such as Hg, Cd, and Pb to determine the uptake of the metals by the plants. Although the soil was contaminated with high concentrations of various metals includes Hg, the

vegetables accumulated was relatively low. The Hg tested in our trial was at a lower level. In addition to the plant species, other factors such as the speciation of the metal and its availability can affect the transfer of the metal thus the accumulation in the plant (Fernández-Martínez et al., 2015). Also, the bioavailability of the Hg in the water, and the physicochemical factors may play an important role to bioaccumulated in the top part of the lettuce. Yu et al. (2018) used a meta-analysis study about Hg uptake factors by different vegetables in soil; they stated that soil pH at acidic levels (pH<6.5) increased the uptake of the Hg by the plants while it decreases at higher levels (pH>7.5). The pH levels of the water in our study were above this level for the first four weeks then decreased to pH=7.3, and 7.0 in the fifth and the sixth week, respectively. Therefore, the pH levels of the study may also affect the bioaccumulation of the Hg in the shoot tissue.

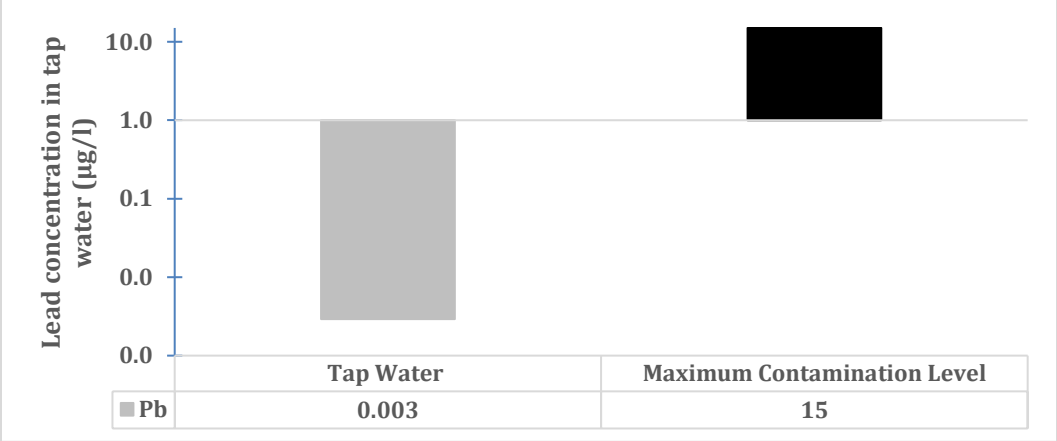
The water of the treatment spiked with a low level of the Pb (1.0% of the MCL=15 µg/L; 0.62 µg/L) as ($C_4H_{10}O_8Pb_3$). The last day data of Pb concentrations in the water, in general, showed no tending to accumulate, and there was no difference compared to the control (Fig 4) ($p > 0.05$) (Table. 5). Compared to the first week, the concentration of Pb decreased in the treatment by the last week. However, the Pb fractions in the water reflected in the fish tissue and the plant root tissue will be discuss later.

The Pb concentration in the fish dry weight converted to the wet weight to compare with the standard of FAO/WHO (Fig. 8) where it was within the standard limit. According to Schenone et al. (2014), heavy metals uptake into the fish tissue

mostly depending on their concentration in the water; which may highly depend on the metal in the fish feed and the feeding ratio. Although the Pb was at low levels in the background water (.003 per 15 µg/L of the MCL) (Fig. 33), and also in the fish feed (0.33 µg/g) compared to the arsenic (Fig. 30), it bioaccumulated significantly in the fish tissue (wet weight) compared to the control ($P < 0.05$) (Table 9). However, the metal was within the MAL (104.1 per

300 ng/g) (FAO/WHO, 1995).

Figure. 33. The lead background concentration in tap water compared to the MCL based on the EPA standard (EPA, 2018).



Martins et al. (2011) studied the effect of Pb concentrations and other metals such as As, and Cd in the water culture of RAS system and on Nile tilapia (*Oreochromis niloticus*) tissue when they used three RAS treatments with different rates of daily water exchange (30, 70 and 1500 l/kg feed/d).They found some metals concentrations such as As, Fe, Mn, Ni, and Zn increased in the water as water exchange rates decreased, but Pb was below detection limit over the study period (71 days). They expected that the accumulation of the metals in the

water might originated from the fish feed. Although the Pb levels were below the limit of detection, it accumulated significantly in the fish liver, but not in the muscle. However, the result of their experiment showed Pb levels accumulated significantly in the liver of the fish, but it was within the permissible level in fish tissues. The result of this study and ours may indicate the tendency of Pb to be accumulated in the fish tissue even at very low levels. Deviller et al. (2005) cultured fish (European sea bass) in different culturing systems. After one year, they found Pb was significantly higher in the fish liver in the RAS compared to the fish cultured in the flow–through system (FTS) where the exchanging water flow rate was constant (on average, 60 times higher than in RAS). This result may show the higher potential for the metal to be bioaccumulated in the fish when the system has less dilution. In our study, the Pb concentration was significantly higher in the treatment comparing to the control by the last day. Therefore, it can be suggested that the bioaccumulation of Pb in the fish tissue may mainly depend on the feeding ratio and its concentration of the metal then on the reuse of the water over the time; the fish feed may be contributed to increasing the availability of the metal to the fish in the water.

The Pb concentration in the plant samples (the shoot tissue) also converted based on the wet weight to compare the standard of FAO/WHO. Its level in the treatment was way below the MAL (0.005 per 0.3 µg/g) of the FAO/WHO standard (FAO/WHO, 1995) (Fig. 15), and there was no difference between the control and the treatment ($P > 0.05$) (Table. 16). The average concentration of the

metal in the treatment at the last week was similar to initial concentration (0.003 µg/g). On the other hand, Pb accumulated in the root (dry weight) of the lettuce in the treatment by the last day of the trial compared to the control (3.32 per 1.32 µg/g) (Fig. 16); the concentration of the Pb decreased in the root of the control by the last day of the trial, but it increased significantly in the treatment compared to the control ($P < 0.05$) (Table. 17).

Our results agreed with some of the results of the study done by Michalska and Asp. (2001) who studied the uptake of Pb and Cd by lettuce (*Lactuca sativa*) in hydroponic culture experiment with different concentrations of Cd and Pb, and they found Pb increased and accumulated more in the plant as its concentration increased in the solution and most of the accumulation located in the root area. Cobb et al. (2000) determined the concentrations of Pb and other metals (Cd, As, and Zn) in different plant tissues including lettuce (Iceberg lettuce) cultured in mine wastes, and in soils mixed with mine wastes in five different treatments (0, 25, 50, 75, and 100% mine tailing). The study's result showed that the Pb concentrations in the root area of the lettuce and the other plants (bean and tomato) were higher than the concentrations in the shoot; they suggested that this result due to the fact that Pb binds to root surfaces and cell walls which can restrict its translocation to the shoots. Also, the Pb concentration was higher and bioaccumulated in the root of the treatments (50, 75, 100%) more than the control. In our experiment, we have similar results when the Pb concentrations were increased more in the roots than the shoots. Also, the Pb increased significantly in the root of the treatment compared to the control. However,

there are some exceptions where the metal bioaccumulated in the root of our experiment at a lower level compared to their study, and also we used a hydroponic system while they cultured the plant in soil.

While many studies mentioned that metals tend to accumulate in the sediments and it is represented as a storage of the heavy metal in an aqua environment, unexpectedly, all metals decreased in the sediments by the last day of the trial period except for the Pb which increased significantly in the treatment (Fig. 20) compared to the control ($P < 0.05$) (Table. 21). The sampling of the sediments could not be done in the first two weeks because we established new aquaponic systems. However, the first samples were able to be collected in the week 3. Despite the peak of the all metals accumulated was at week 4, but their concentrations decreased later until the last week of the trial. Unlike the results in Pb, there was no significant difference between the control and the treatment of the other HMs (As, Cd, and Hg).

It can be suggested that the decreasing of the HMs concentrations in the sediments may be due to several factors such as pH, the aerobic condition, the flow rate of the water in the system, and the adsorption to the bacteria biofilms (bio-balls in the filters).

Ikem et al. (2003) stated that in addition to the quality of physical and chemical factors, releasing heavy metals in the water from sediments rely on the several factors such as metal speciation, sediment pH and of the aquatic system. Eggleton and Thomas (2004) stated that the chemical properties of sediment could be affected when it disturbed thus this can induce the contaminants

mobilization; however, few articles studied this phenomenon, and the factors affecting the release of the contaminants from sediments still not completely understood. Changing the pH and the redox potential (Eh) can accelerate desorption, oxidation, and partitioning of the contaminants from sediments thus affecting the affinity in sediments and on their bioavailability (Eggleton and Thomas, 2004). Li et al. (2013) investigated the effects of pH, temperature, dissolved oxygen over the time, and flow rate of overlying water on releasing of heavy metals such as Cd, Pb, Zn, Cr, and Cu from storm sewer sediments; they found the rate of the metals releasing was greater in low pH levels (4-7) compared to (8-10) levels. Moreover, in general, increasing of the water temperature (30–35°C) comparing to the lower temperatures (15, 20, 25 °C), dissolve oxygen from (1 to 9 mg/L) over 350 min, and the flow rate increased, in turn, the releasing of the metals from the sediments.

In our experiment, the pH level of the treatment decreased from 8.2 in the first week compared to 7 by the last week. Also, the dissolved oxygen levels where in between (6-6.3 mg/L), but the water temperature was at 25.4°C during the experiment period. However, although we did not measure the flow in the system, the concept of aquaponic systems are based on the permanent flowing of the circulating water which may disturb the sediment. Also, the sediments collected in the biofilter which contains many small plastic bio-balls, for increasing the surfaces for the bacteria, which may increase the disturbance of the sediment over the water flowing along the time. According to Ikem et al. (2003), due to

remobilization, metals can recirculate and redistribute within the water column and thus to fish; water currents can disturbance the sediments into the water column which may re-mobilize the metals (Burridge et al., 2010).

The metals concentrations started to decrease after the 4th week of the experiment where the pH range of the control and the treatment decreased from 8.2 in the first week to 7.0 in the last week which make the metals more available in the water to plants and fish t. According to Burridge et al. (2010), if metals decreased in sediments, they may release back into the water column, and therefore, they can be more available to the organism of the water body. Pb levels in the study of Li et al. (2013) increased rapidly under the aerobic condition, but this had an opposite effect on Cd releasing. In addition, the flow rate significantly affected the releasing of Pb, Zn, and Cr, but did not (slightly) on Cu and Cd. Similarly, in the current experiment, only Pb significantly increased in the water compared to the control over the study period.

The distribution of the HMs in the components of the system is differ. However, all the HMs, except for the As, tend to concentrate in the sediments, more precisely, at the bacteria biofilm on the bio-balls due to the highly adsorption properties between the metal ions and the bacteria cell wall or their biofilm communities. On the other hand, the availability of the As was mostly in the water samples of the treatment.

The tendency of the HMs distribution in the components of the system were higher in the bio-balls for all metals except for As were tended to concentrate in the water; The HMs tendency to accumulate on the bio-balls were on the following order, Hg (>99%), Pb (>85%), Cd (>58%), and As (>29%).

As concentrated higher in the system components in the following order: water> bio-balls> root> sediment> fish> shot at 45%, 28%, 9%, 8%, 6%, 1%, respectively. For Cd, Bio-balls> Sediment> root> shoot> water> fish at 85%, 29%, 5%, 4%, 1%, and 0.4%, respectively. For Hg, Bio-balls> fish > Sediment > root > shoot> water at 99%, 0.1%, 0.07%, 0.06%, 0.06%, and 0.01%, respectively. For Pb, Bio-balls> Sediment > root > fish > shoot> water at 85%, 13%, 1%, 0.13%, 0.04%, and 0.02%, respectively.

Figure. 34. Concentration and percentage of As distribution in the components of the treatment system

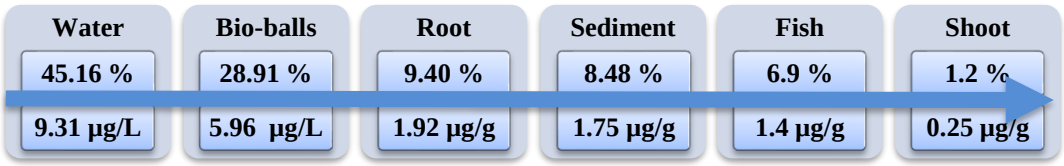


Figure. 35. Concentration and percentage of Cd distribution in the components of the treatment system

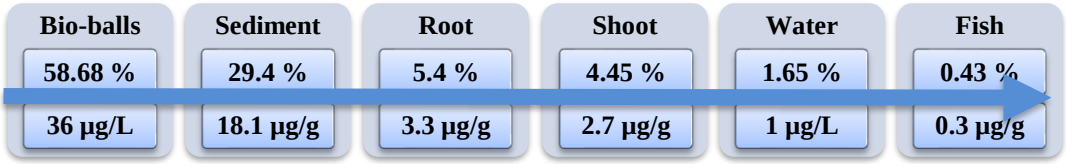


Figure. 36. Concentration and percentage of Hg distribution in the components of the treatment system

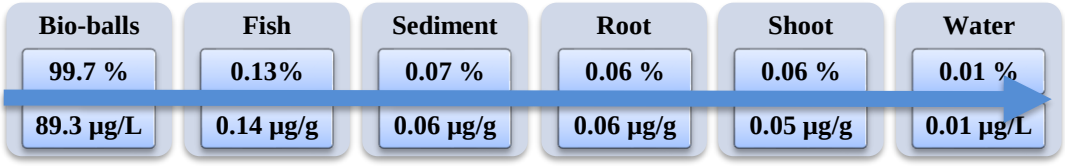
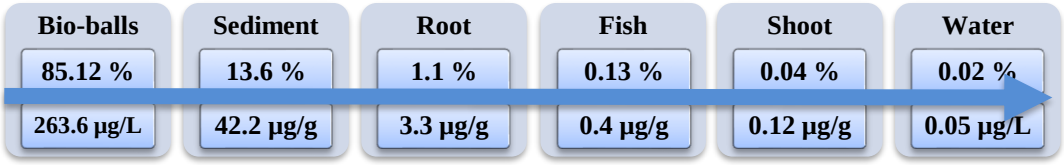


Figure. 37. Concentration and percentage of Pb distribution in the components of the treatment system



Conclusion

Only As accumulated in the water during the trial period. Although As, as average, was within the MCL. The order of the most concentration of the HMs by the last week in the water was As>Cd>Pb>Hg. In the fish tissue, as wet weight, only Hg and Pb have MAL set by the FAO/WHO standards. Compared to the control in the first day, both metals accumulated by the last day of the experiment. However, they were within the MAL. The order of the greatest accumulation of the HMs in the fish tissue, as dry weight, was as the following: As> Pb> Cd>Hg. The leafy vegetables MAL set by the FAO/WHO included Cd and Pb. Both metals compared with the standards after converted the concentration of the metals as the dry weight to the wet weight. The Cd concentration was about one third of the MAL while the Pb levels were way below these limits. The data analyzed of the HMs concentrations in the lettuce shoot, as high average levels of the dry weight, were in the following order: Cd>As> Pb>Hg. On the other hand, All HMs bioaccumulated in the root tissue of the lettuce in the treatment by the end of the experiment except for Cd. The order of higher metals concentration in the root as follows Pb≥Cd>As>Hg. Finally, no metal accumulated in the sediment except for Pb where increased significantly compared to the control by the end of the trial. The order of the high concentrations of the HMs reported of the treatment in the sediment by the last week were: Pb>Cd>As> Hg.

Annex 1

Nitric acid (HNO3) diluting

Lab equipment that used many times such as the graduated glass cylinder and the forceps are cleaned by rinsing before and after each sample using a plastic wash battle contained a diluted HNO_3 solution with Dsw. About 63.7 ml of Nitric acid (70%) added into a 250 ml distilled water then diluted into 1000 ml of distilled water.

FIGURES

Figure. 38. Daily air temperature values (C°) during the experimental period.

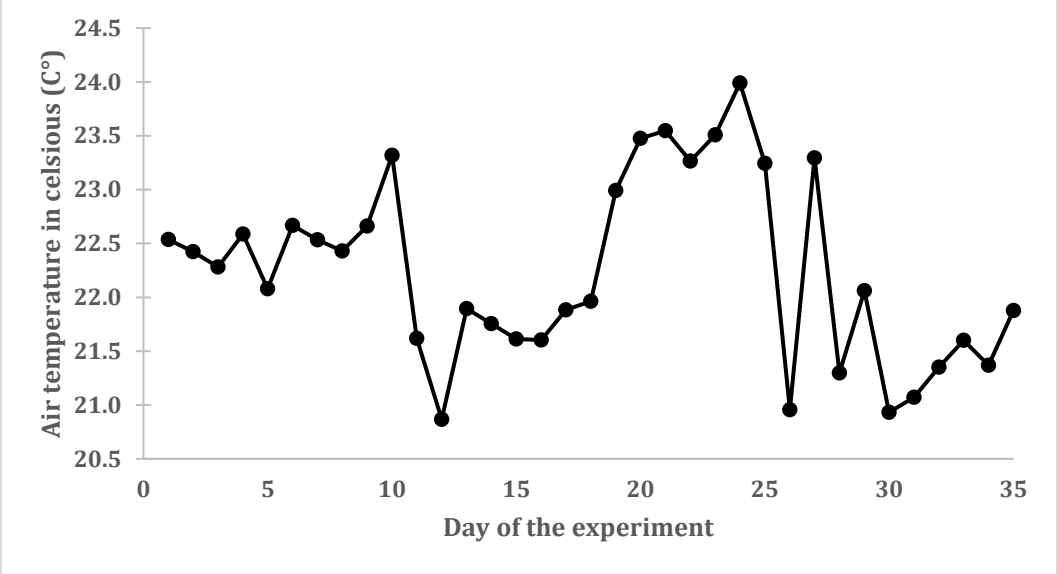


Figure. 39. Daily relative humidity percentage values (%) during the experimental period.

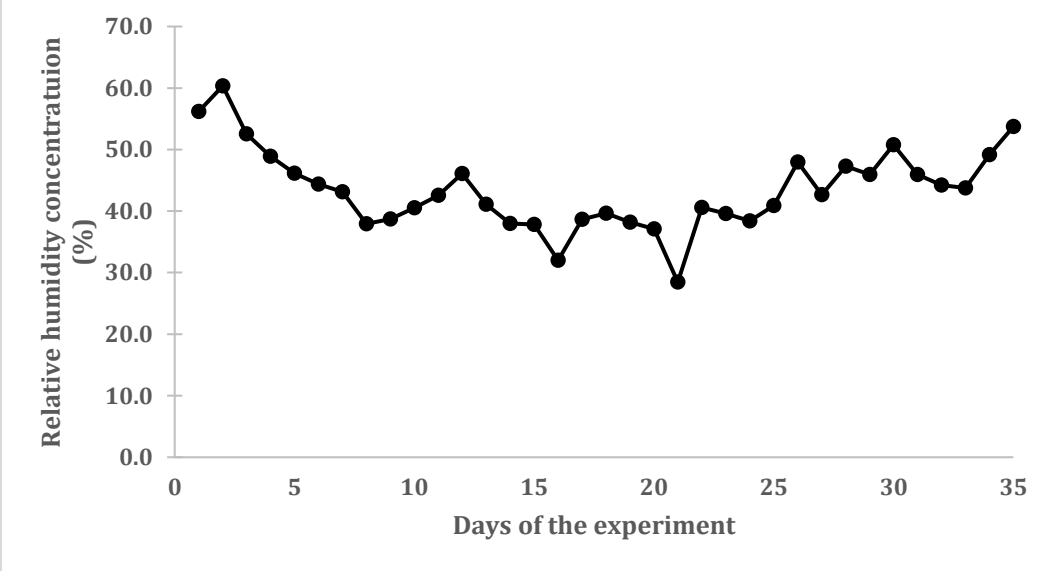


Figure. 40. Daily photosynthetic active radiation (PAR) values (µmole/m^2/s) during the experimental period.

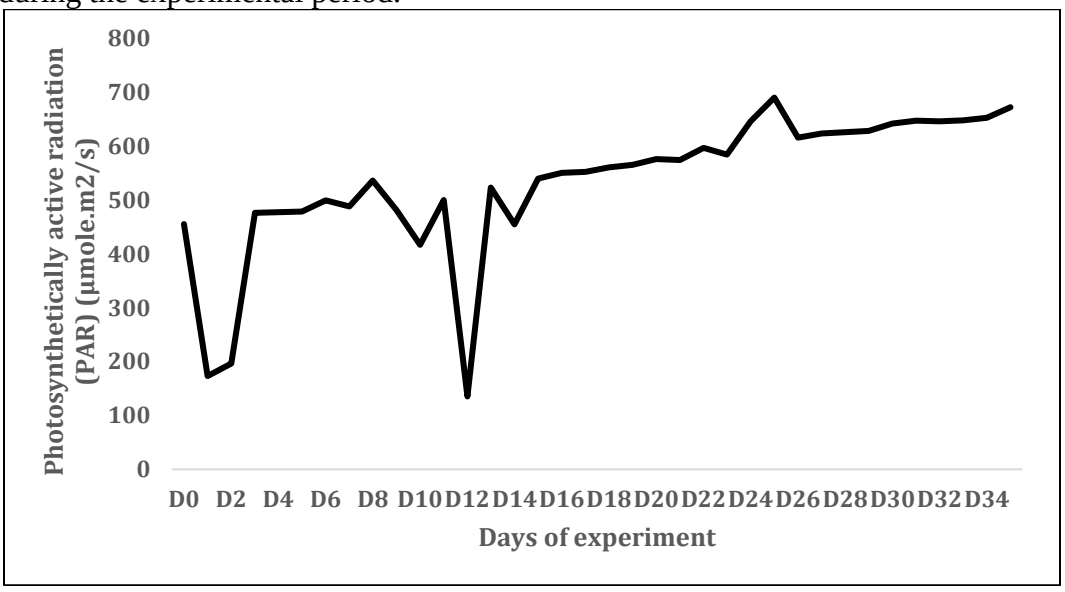
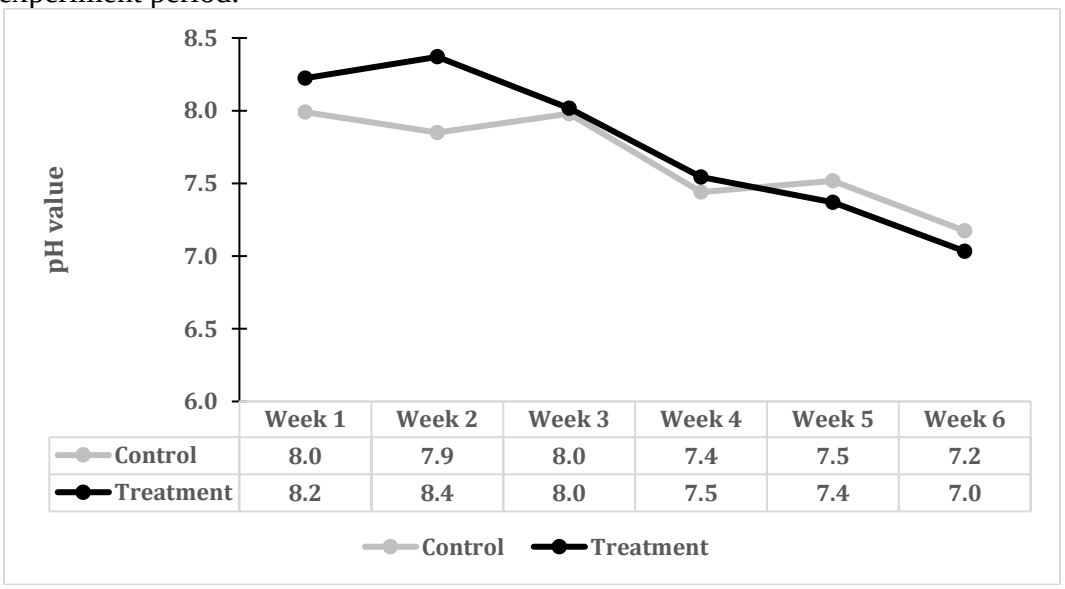


Figure. 41. Weekly average pH values (control and treatment) during the experiment period.



	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control	8.0	7.9	8.0	7.4	7.5	7.2
Treatment	8.2	8.4	8.0	7.5	7.4	7.0

Figure. 42. Weekly average DO (mg/L) values (control and treatment) during the experimental period.

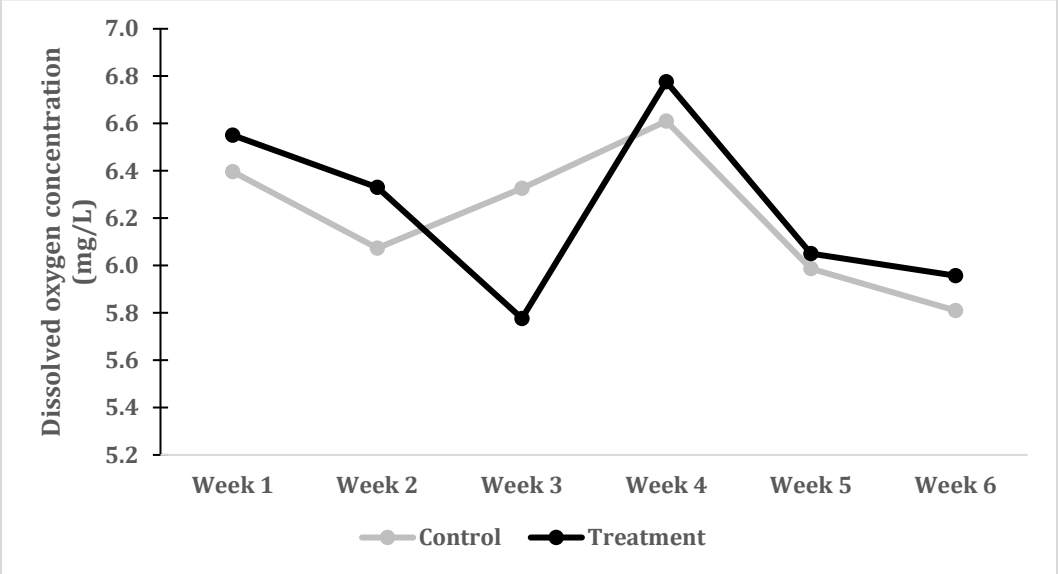


Figure. 43. Weekly average EC values (control and treatment) during the experiment period mS/cm.

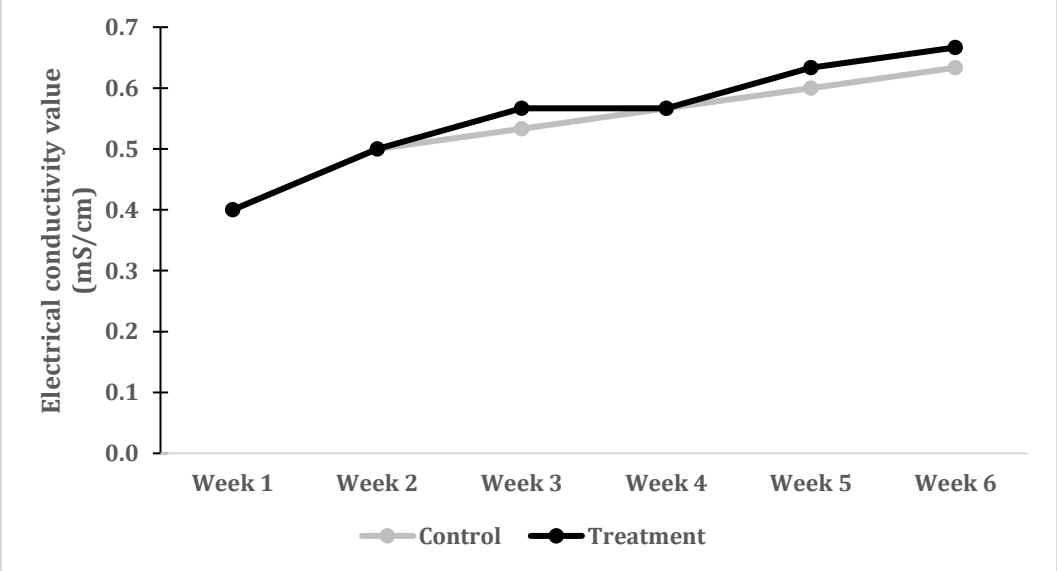


Figure. 44. Weekly averages of water temperatures (Tw) (C°) of the control and the treatment during the experiment period.

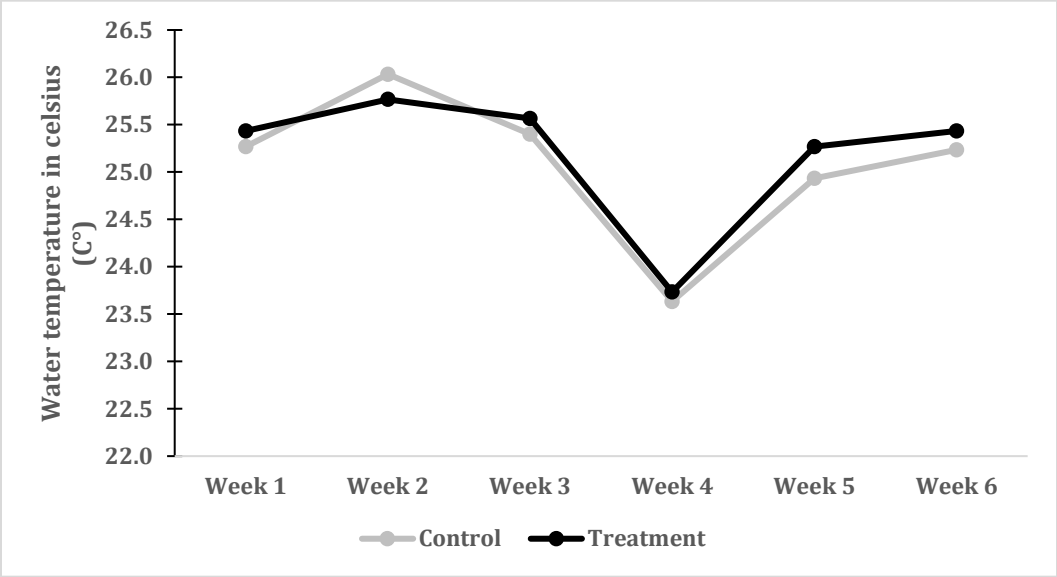


Figure. 45. Weekly average of ammonia (NH_3) values (mg/L) (control and treatment) during the experiment period.

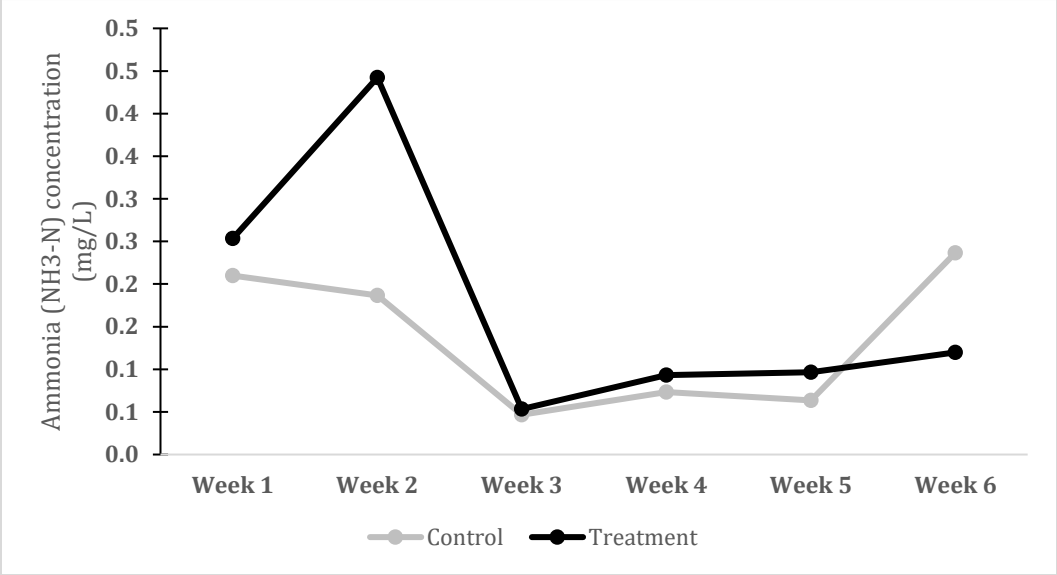
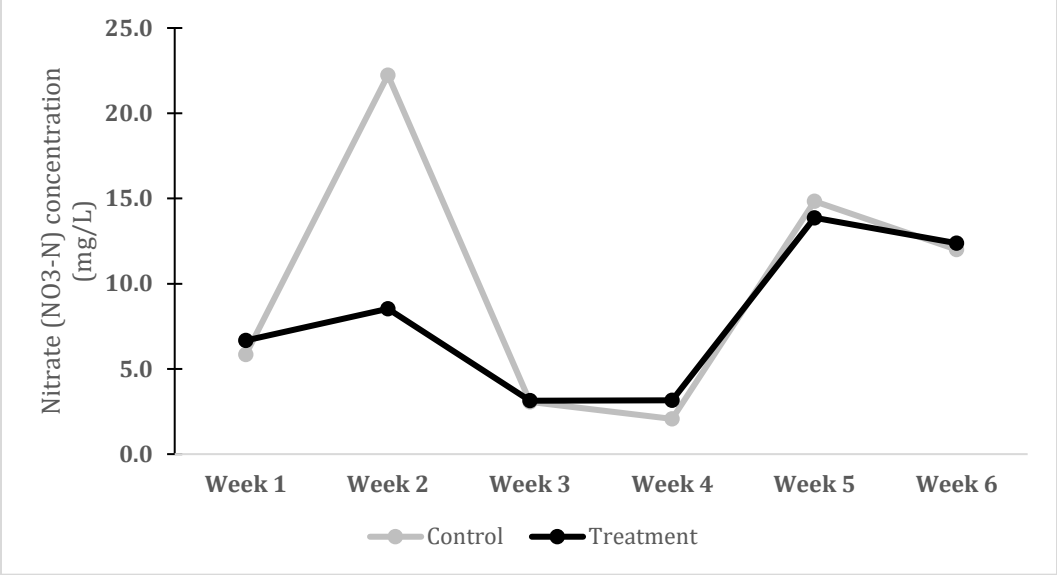


Figure. 46. Weekly average of nitrate (NO_3^-) values (control and treatment) during the experiment period.



TABLES

Table. 23. The average of arsenic concentration of the control and the treatment in the water (µg/L). P-value (means of the control and the treatment). The standard deviation (S.d) of the means. MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
Week 1	0.01	0.01	1.92	0.48	0.016
Week 2	0.78	0.53	2.86	0.26	
Week 3	1.36	0.47	3.61	0.45	
Week 4	1.86	0.67	4.11	0.47	
Week 5	6.06	0.59	8.85	0.59	
Week 6	6.21	0.60	9.31	0.72	
MCL	10				µg/L

Table. 24. The average of mercury concentration of the control and the treatment in the water (ng/L). P-value (means of the control and the treatment). The standard deviation (S.d) of the means. MCL for the arsenic in the drinking water based on the U.S EPA standard (EPA, 2018).

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
Week 1	4.83	3.53	35.75	4.77	0.04
Week 2	17.12	6.62	19.35	1.14	
Week 3	10.05	2.57	9.18	0.38	
Week 4	11.28	1.35	12.05	1.27	
Week 5	5.96	0.49	7.97	0.53	
Week 6	6.05	0.45	7.20	1.10	
MCL	2000				ng/L

Table. 25. The average concentration of mercury (wet weight) (ng/g) for the control and the treatment in the fish tissue. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard (FAO/WHO, 1995), and the standard deviation (S.d) of the means.

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
First day	3.58	0.15	3.63	0.09	0.006
Last day	9.95	1.00	29.86	2.99	
MAL	500				ng/g

Table. 26. The average concentration of lead (wet weight) (ng/g) for the control and the treatment in the fish tissue. P-value (means of the control and the treatment) with the MAL by FAO/WHO standard (FAO/WHO, 1995), and the standard deviation (S.d) of the means.

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
First day	38.65	1.57	39.19	0.96	0.006
Last day	29.59	9.33	104.09	14.42	
MAL	300				ng/g

Table. 27. The average concentration of arsenic (dry weight) (µg/g) for the control and the treatment in the root tissue of the lettuce. P-value (means of the control and the treatment) with the standard deviation (S.d) of the means.

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
First day	0.51	0.00	0.51	0.00	0.016
Last day	0.81	0.13	1.34	0.06	

Table. 28. The average concentration of lead (wet weight) (µg/g) for the control and the treatment in the root tissue of the lettuce. P-value (means of the control and the treatment) with the standard deviation (S.d) of the means.

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
First day	1.93	0.00	1.93	0.00	0.023
Last day	0.87	0.41	2.30	0.38	

Table. 29. The average concentration of lead (µg/g) for the control and the treatment in the sediment over the last four weeks of the experiment with the P value and the standard deviation (S.d) of the means.

	Control		**Treatment**		**P-value**
	M±	S.d	M±	S.d	
Week 3	1.79	0.48	10.52	1.38	0.024
Week 4	2.63	0.96	22.81	14.77	
Week 5	0.49	0.28	2.81	1.73	
Week 6	0.43	0.15	3.87	1.08	

Table. 30. Instrumentation parameters for ICP-MS (provided by ALEC laboratory)*.

RF power (w)	1450
Dwell time (ms)	50
Sweeps per replicate	100
No. of replicates	3
Acquisition mode	Peak hopping
Argon flow rates (L/min):	
Nebulizer flow	0.95
Coolant	15
Auxiliary	1.3
Sample uptake (ml/min)	~0.400
Presence of oxides as CeO/Ce	< 3%
Presence of doubly- charged species (as Ba^{++}/Ba)	< 3%
Nebulizer type	Micro-mist
Spray chamber	Scott Double-pass quartz
Sample and Skimmer cones	Ni

*provided with permission from ALEC lab, University of Arizona, Tucson, Az, U.S.

Table. 31. Photosynthetically active radiation (PAR) (µmol·m-2·s-1) of the study period from 8th Jan – 12 Feb, 2018.

Year	Date	Time	PAR
2018	8-Jan-18	1200	455.4
2018	9-Jan-18	1200	173.2
2018	10-Jan-18	1200	196.6
2018	11-Jan-18	1200	476.3
2018	12-Jan-18	1200	477.8
2018	13-Jan-18	1200	478.7
2018	14-Jan-18	1200	499.3
2018	15-Jan-18	1200	488.4
2018	16-Jan-18	1200	536.4
2018	17-Jan-18	1200	481.6
2018	18-Jan-18	1200	417.1
2018	19-Jan-18	1200	499.9
2018	20-Jan-18	1200	135.4
2018	21-Jan-18	1200	523.5
2018	22-Jan-18	1200	454.9
2018	23-Jan-18	1200	540.2
2018	24-Jan-18	1200	550.4
2018	25-Jan-18	1200	552.3
2018	26-Jan-18	1200	560.6
2018	27-Jan-18	1200	565.6
2018	28-Jan-18	1200	576.3
2018	29-Jan-18	1200	574.7
2018	30-Jan-18	1200	597.3
2018	31-Jan-18	1200	584.6
2018	1-Feb-18	1200	646.5
2018	2-Feb-18	1200	690.4
2018	3-Feb-18	1200	616.2
2018	4-Feb-18	1200	623.6
2018	5-Feb-18	1200	626
2018	6-Feb-18	1200	628.7
2018	7-Feb-18	1200	642.4
2018	8-Feb-18	1200	647.7
2018	9-Feb-18	1200	646.2
2018	10-Feb-18	1200	648.1
2018	11-Feb-18	1200	653
2018	12-Feb-18	1200	672.6

Table. 32. Comparison of the maximum contamination limit (MCL) of the U.S. Environmental Protection Agency for the HMs in the drinking water and the maximum allowable limit (MAL) of the Food and Agriculture Organization/World Health Organization Expert Committee (FAO/WHO)

Metal	MCL (µg/L)*	MAL (µg/g)**	
		Fish	Lettuce shoot
Arsenic (As)	10 µg/L	NA	NA
Cadmium (Cd)	5 µg/L	NA	0.2
Mercury (Hg)	2 µg/L	0.5	NA
Lead (Pb)	15 µg/L	0.3	0.3

*water samples
**Wet weight samples of the fish and the plant shoot

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APPENDIX B - EFFECT OF HEAVY METALS ACCUMULATION ON BACTERIAL ANTIBIOTIC RESISTANCE IN AN AQUAPONIC SYSTEM

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ABSTRACT

This study was done to evaluate the accumulation of some toxic heavy metals and metalloids (Cd, Pb, Hg, and As) (HMs) on the potential co-selection for bacterial antibiotic resistance (BAR) to ampicillin and tetracycline antibiotics in an aquaponic system over a short experimental period (35 days). The co-selection may occur due to nonantibiotic compounds such as metals which may promote antibiotic resistance through co-resistance or cross-resistance mechanism. Aquaponics is a technology that holds promise to enhance global food production. However, in addition to direct food safety hazards, HMs can become more concentrated and could conceivably present a potential human health risk due to the BAR. The resistance of bacteria to ampicillin and tetracycline was investigated in the aquaponic system after it was spiked with some toxic metalloid and heavy metals (arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)).

BAR was also measured in a control system that did not intentionally receive any HMs. Each treatment was replicated in triplicate, with each replicate stocked with 25 Tilapia (*Oreochromis niloticus*) fingerling fish and six plants (Butterhead lettuce (*Lactuca sativa*)). The treated systems received mixed HMs with concentrations of As, Cd, Hg, and Pb of 20%, 15%, 1.5%, and 1.0%, respectively of the maximum contaminant level (MCL) of each HM set by the United States Environmental Protection Agency for drinking water (EPA, 2018). Weekly water samples were collected to evaluate levels of bacterial antibiotic resistance starting on the first day of the experiment and extending to the end of the experiment period. The results showed that ampicillin-resistant bacteria decreased in both the

control and the treatment system over the length of the experiment. Tetracycline-resistant bacteria increased until the third week of the treatment, then decreased gradually until no resistant bacteria colonies were found in samples collected during the final week in the treatment as well as in the control. The outcome of this study can aid the sustainability of the aquaponic industry through ensuring the safety of the products for consumers.

Introduction

Bacterial Antibiotic Resistance

Antibiotic resistance is defined as the ability of microorganisms to overcome the effects of antimicrobial agents (Li and Webster, 2018). Bacterial antibiotic resistance (BAR) is not a new emergent issue, but it is ancient. For instance, bacteria isolated from ancient ice cores from the Antarctic show resistance to different levels to antibiotics and metals (De Souza et al., 2006). Infections caused by antibiotic-resistant bacteria are rising worldwide (Seiler and Berendonk, 2012). Antibiotic-resistant infections are a serious health issue due to the difficulty of treatment, and patients may need extended treatment in hospitals, which increases health costs (CDC, 2017).

Metal/loids such as (As, Hg, and Cu) have antimicrobial activity against bacteria, and they used before the widespread use of antibiotics. The widespread production and use/missuses of antimicrobials agents such as antibiotics and metals has developed and increased the opportunities for selection the bacteria resistance. Bacteria resistance can be acquired through mutation or by obtained resistance genes from other bacteria. They can carry the resistance genes through mobile genetic elements that able to transferring in the environment that may spread this ability to other bacteria that had not the resistant property in the past. Resistance genes can be transferred horizontally in different environment to other bacteria by three mechanisms: conjugation, transformation, and transduction (Pal et al, 2017). Therefore, bacteria can become more resistant over time.

Extensive use of antibiotics may increase the risk of emergence, development, and spread of antibiotic resistance due to increased selective pressure on bacterial populations and can ultimately lead the drugs to be useless (Burridge et al., 2010; Zhang et al., 2009). According to national reports such as those from the Institute of Medicine (IOM), National Institutes of Health (NIH), Food and Drug Administration (FDA), and Centers for Disease Control and Prevention (CDC), drug-resistant bacteria are posing a severe threat to human health. Drug-resistant and harmful bacteria have multiplied and spread at worrying levels, and because bacteria have developed a resistance to antibiotics, infections have gotten more difficult to treat (Bax and Griffin., 2012). As reported by the World Health Organisation (WHO), high levels of antibiotic resistance widespread occurrence worldwide; each year, many millions of people are infected with resistant bacteria in many developing and developed countries (Li and Webster, 2018; Lindmeier, 2018). This situation has limited the choices of antibiotic treatment infection and has greatly increased health care costs; for example, In the U.S., 2 million antibiotic-resistant infections lead to 23,000 deaths every year, resulting in $55– 70 billion yearly economic costs (CDC, 2017). Early discovery of antibiotics improved the quality of life worldwide; for instance, when penicillin was discovered in the early 1900s, it was successfully used to treat patients in World War II who may have died otherwise (Li and Webster, 2018).

Misuse and overuse of antibiotics has increased their release into the environment over past decades, with disturbing consequences (Di Cesare et al., 2016; Suzuki and Hoa, 2012). For example, antibiotics used to control human

bacterial infections are also routinely used to control the growth of potentially harmful microorganisms during transcontinental transport of ornamental fish (Verner-Jeffreys et al., 2009; Cole et al., 1999). According to Cole et al. (1999), because of the wide use of antibiotics, several indications mentioned that some bacteria have developed to antibiotics such as tetracycline. Overuse of antibiotics, with weak regulations in many countries, has helped to boost the resistance in many strains of pathogens (Percival et al., 2014; Burridge et al., 2010). Many antibiotics are not broken down completely in the body, and due to their stability, they can reach to the environment through waste products; therefore, the use of antibiotics has increased concerns for environmental loading of these contaminants due to the extensive worldwide use. (Burridge et al., 2010).

Although antibiotics are active agents used for curing people, they are also used for agricultural purposes such as preventing and treating infections of animals and plants or as growth promoters in animal farming as feed additives (Martinez, 2009; Zhang et al., 2009). Animal farms contribute in the spread of the BAR through the potential residues of antibiotics and antibiotic resistance genes that animal farm may contain. In recent years, since 2003, antibiotics have been banned in the marketing and to be used for promoting the growth in Europe (Santovito et al., 2018) and in the U.S since 2017 (McKenna, 2017). Quinolone antibiotics, a large group of broad-spectrum bactericides were used in many countries to treat bacterial infections, including Canada, Scotland, and the United States, for treating fish. They are banned at present in these countries due to their

risks to disseminating BAC because quinolones resistance were identified in marine bacteria and human pathogens as well (Burridge et al., 2010).

The emergence of antibiotic resistance genes in the water environment is a rising global concern (Zhang et al., 2009); unfortunately, it is common to find surface water polluted with antibiotics. Avoiding contamination with these pollutants is difficult except for pristine sites less affected with the human activities (Zhang et al., 2009). Discharge of antibiotics into the environment is often linked with entry of antibiotic-resistant organisms into water body (Baquero et al., 2008). Antibiotic resistance genes and resistant organisms have been isolated from not only medical wastewaters, and agriculture production wastewaters, but also from wastewater treatment plants, sewage, surface water, groundwater, and also from drinking water (Zhang et al., 2009). Antibiotics have also been found in the rivers and tap water in Spain and China (Huang et al., 2015; Valcarcel et al., 2011).

The quality of human food can directly be affected by the presence of residual antibiotics in farmed fish (Burridge et al., 2010). Using antimicrobials, such as antibiotics including quinolones, and tetracyclines, for controlling pathogenic infections in aquaculture is a common activity (Defoirdt et al., 2007) in certain countries. There are only a limited number of antibiotics that have been used or approved for use in food fish in developed countries. There are many examples of antibiotics that may be used in aquaculture. For example, *Amoxicillin Trihydrate* for Atlantic salmon, *Ampicillin* for aquarium fish, *Erythromycin* used at different stages in fish life cycle, *Florfenicol* for treating rainbow trout fry syndrome, and

Oxytetracycline as effective antimicrobial agent controlling several fish pathogens (Noga, 2010). In addition, using antibiotics such as tetracycline in commercial fisheries since the 1990s is a widespread practice during transferring fish overseas for trade purposes to control the growth of potential pathogens (Verner-Jeffreys et al., 2009; Cole et al., 1999). For instance, quinolone antibiotics, which inhibit DNA replication of bacteria, are used in sea fish such as salmon for treating Gram-negative bacteria including *P. salmonis*, a pathogen that infects salmon, *Furunculosis,* a bacterial infection in aquaculture industry, and in marine bacteria such as *Vibrio* and *Aeromonas* (Burridge et al., 2010).

The use of antimicrobial agents in aquaculture potentially selects for microorganisms antimicrobial-resistant in the marine environment. This is because susceptible bacteria are killed by the antibiotic, while resistant organisms survive and replicate. Tomova et al. (2018) reported that the extensive use of quinolone antibiotics, a large group of broad-spectrum bactericides, resulted in identification of quinolone-resistance genes such as qnrA, qnrB, and qnrS in the chromosomes of randomly-selected bacteria. Discharging a residue of antibiotics in farm wastes could conceivably increase environmental bacterial resistance (Suzuki and Hoa, 2012). In turn, resistance genes can transfer horizontally among and between species of bacteria. Such spread of resistance can be more pronounced in aquatic environments. For example, the ability to potentially inactivate amoxicillin using β lactamases is a property shared by both fish and human pathogens (Burridge et al., 2010), increasing the potential for passage of antibiotic resistance through the human food chain

In bacterial communities, studies have reported correlations between spreading and persistence of bacterial antibiotic resistance genes and anthropogenic activities responsible for releasing antibiotics and antibiotic-resistance genes in surface waters (Di Cesare et al., 2016). Over the past 20 years, antibiotics such as *amoxicillin*, *florfenicol*, *tribrissen*, *tetracycline*, and *Erythromycin* have been used in aquaculture for inhibiting infections of pathogenic bacteria. *Amoxicillin*, for example, is a broad-spectrum antibiotic that works by disrupting bacterial cell wall biosynthesis, has been widely used to treat fish infected with Furunculosis bacterial disease. *Oxytetracycline*, an antibiotic used for Furunculosis and Vibriosis infections, controls bacterial growth by inhibiting protein synthesis (Burridge et al., 2010).

Bacteria adaptation mechanism of resistance

Based on the process of natural selection, bacteria, like any other organism, can evolve and adapt to their environment, and can pass on evolved traits to further generations.

Bacteria can acquire resistance against antibiotics either by mutation or through the transferring of genetic elements that encode resistance to the antibiotics; transfer of genes (known as horizontal gene transfer) can occur between the same bacteria group or between different species (MacGowan and Macnaughton, 2013). Horizontal gene transfer occurs as bacterial conjugation, transformation, and transduction; each of these represent mechanisms whereby bacteria disseminate the antibiotic resistance genes to other bacterial strains

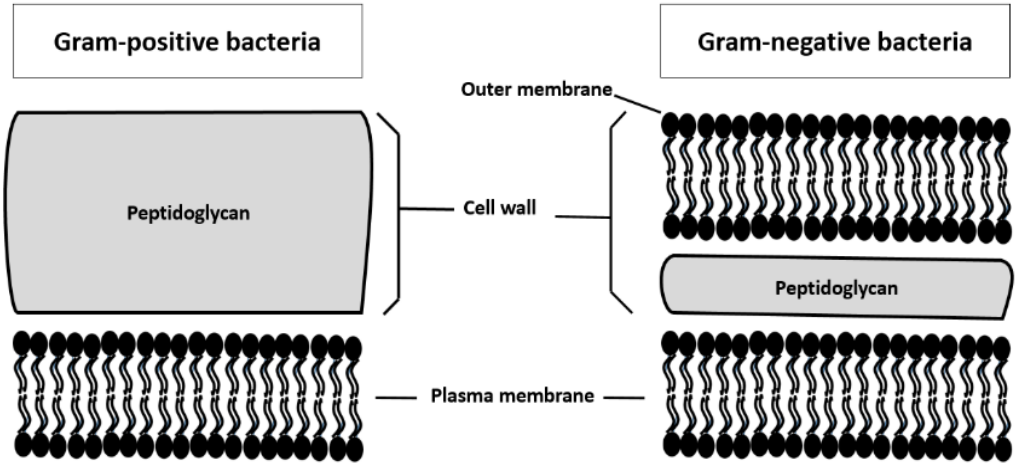
(Munita and Arias, 2016; Ilina et al., 2013).

The conjugation is the process of transfer genetic materials or bacterial genes of one bacterium to another by direct contact (Singer et al., 2016). The process of transformation includes the release of naked DNA followed by uptake of the DNA by another bacterium and recombination of the DNA into the new genome (Thomas and Nielsen, 2005) so it is based on the bacterial acquisition of naked DNA from the surrounding environment (Singer et al., 2016). Transduction occurs when the gene or the DNA introduced into bacteria is mediated by bacteriophage, a virus that infects and replicates within a bacterium (Singer et al., 2016; Di Cesare et al., 2016).

Resistance to antibiotics conferred by chromosome mutations or transposable elements can be the result of one of four cellular mechanisms. The first mechanism involves a reduction in membrane permeability, which in turn, reduces antibiotic access. Second, an antibiotic can be inactivated by breakdown or chemical modification of the drug. A third strategy is the mutation, modification, or replacement of the cellular antibiotic target, so the target is no longer sensitive to the antibiotic. The final strategy involves the rapid efflux of the antibiotic, which prevents accumulation of the drug, so the antibiotic cannot reach the effective level (Munita and Arias, 2016; Baker-Austin et al., 2006; Krulwich et al., 2005). An example of the fourth strategy is tet(L), a chromosomally-encoded antibiotic-efflux transporter that encodes a tetracycline efflux protein in *Bacillus subtilis*, a Gram-positive organism (Krulwich et al., 2005).

In addition to the genetically-encoded mechanisms for antibiotic resistance above, bacteria also have intrinsic properties that can reduce the efficacy of antibiotics. For example, biofilms can confer natural resistance to antibiotics by protecting bacterial communities from antibiotic penetration (Stewart, 2002). Also, the general cell wall structure of the bacteria can confer natural resistance. (Fig. 1); Gram-positive bacteria (left) have a thick peptidoglycan layer and an inner phospholipid bilayer membrane that surrounds the cytoplasm of the cell. The outer cell membrane of Gram-negative bacteria is coated with lipopolysaccharides and an inner cytoplasmic membrane separated by a thin peptidoglycan layer, which is bound to the outer membrane by another phospholipid bilayer layer. Penicillin targets a specific protein located in the peptidoglycan, the cell wall layer of bacteria. However, Gram-negative bacteria have unique cell-wall structures which are protected by an extra outer membrane (phospholipid) layer which reduces the entry of penicillin, while this feature is absent in Gram-positive bacteria.

Figure. 1. The differences between the cell-wall structures of Gram-negative and Gram-positive bacteria.



In addition to natural resistance, microorganisms may develop, acquire, and spread antibiotic resistance to other bacteria in various ways. They can acquire resistance to antibiotics from another bacterium through horizontal gene transfer (e.g., by gaining movable genetic elements containing resistance genes from another bacterium) (Li and Webster, 2018; Verner-Jeffreys et al., 2009).

Cellular mechanisms for antibiotic resistance

Bacterial mutation is a change that can occur in the sequence of the bacteria's DNA or genes as the result of selective pressures posed by environmental factors such as heavy metals and antibiotics. Mutations allow the bacteria to gain an advantage to overcome the antimicrobial effects of these factors (Munita and Arias, 2016; Ilina et al., 2013). In the event of a mutation, the susceptible bacterial population is killed, while resistant bacteria multiply and become predominant, despite the presence of the environmental stressor (Munita and Arias, 2016).

Transferring the resistance genes

As described above, bacteria can become resistant to an antibiotic by acquiring external genetic determinants such as plasmids from resistant microbes in the surrounding environment (Munita and Arias, 2016).

Transposable elements, also known as mobile genetic elements, transposon genes, or jumping genes, are sequences of DNA that can migrate from one location to another within a genome, or between genomes of different organisms, and can cause genome changes that can induce resistance to antibiotics (Miller and Capy, 2004; Saedler et al., 1996). Such elements can be present in microbial

communities in large amounts. According to Huang et al. (2016), bacteria resistant to tetracycline accounted for 3.97% or more of the total bacterial community in waste sludge, while tetracycline resistance genes were detected at 10^8–10^{13} copies per gram.

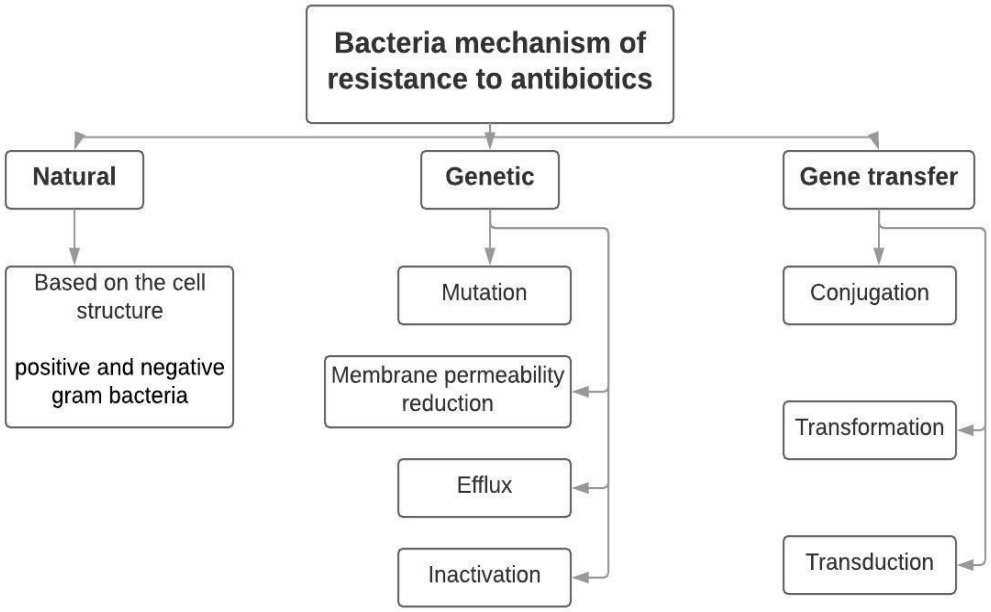
Exogenous genes can be transferred from one bacterium to another via three main strategies; first, conjugation, a simple mating process, where bacteria can share the mobile genetic elements that carry genes encoding resistance to antibiotics by a close relationship with a donor organism. Second, transformation occurs when bacteria uptake naked extracellular DNA released in the environment. This process starts with exposing the recipient bacteria to donor DNA or extracellular DNA molecules that are released from disrupted cells, decomposing cells, or are excreted from living cells. The DNA is incorporated into the chromosome of recipient bacterial cells (Munita and Arias, 2016; Thomas and Nielsen, 2005). The third mechanism is transduction, which involves the transfer of genes by viruses called bacteriophages; the viruses infect and replicate within the bacterial host (Perry and Wright, 2013; Thomas and Nielsen, 2005).

The persistence and spread of antibiotic resistance genes among bacterial communities through acquisition and recombination helps to moving the antibiotic resistance genes into different environments (Suzuki and Hoa, 2012). Anthropogenic activities can help the spread of antibiotic-resistance genes; for example, bacteria within wastewater treatment plants could share genetic elements encoding for antibiotic resistance (Huang et al., 2016), where they could spread resistance upon released into aquatic systems. Also, bacteria can share

genetic determinants in the environment through humans and fish. Genetic determinants for resistance to sulfa and quinolone drugs can be found in human and fish guts (Burridge et al., 2010).

According to Rasmussen and Sorensen (1998), the ability of bacteria to transfer resistance selects for bacterial survival in polluted sites. Their results show that the number of isolates containing resistance plasmids was significantly higher in a contaminated site compared to the unpolluted site. The high transmission of resistance plasmids increased the resistance to Hg as well as to tetracycline in the bacterial community of the polluted site, where 93.5% of the isolates samples contained tetracycline resistance plasmids and 61% contained Hg resistance plasmids. Also, the tetracycline and Hg-resistance genes themselves were higher in the isolates, which indicated a high potential of conjugative plasmids (30% and 29% respectively).

Figure. 2. Acquiring and resistance mechanisms of germs to some antibiotics.



Heavy metals as selective promoters of BAR

The factors that drive the selection for bacterial resistance are still unclear (Di Cesare et al., 2016), but several studies suggest that heavy metals co-select for BAR in environmental bacteria (Zhang et al., 2009). Heavy metals such as Hg, Cd, copper (Cu), and zinc (Zn) may boost the evolution of antibiotic resistance because bacterial tolerance mechanisms for metals and antibiotics can be the same (Baker-Austin et al., 2006). Thus, bacteria located in metal-contaminated environments can easily and commonly obtain antibiotic resistance compared to uncontaminated sites (Zhang et al., 2009; Baker-Austin et al., 2006). According to the study done by Stepanauskas et al. (2006), comparing of samples for bacterioplankton collected from ash settling basins of three coal-fired power plants (intake and discharges) showed significantly more tolerant to metal and antibiotic in discharges samples. In addition to triggering co-selection processes, heavy metal pollutants increase the bacterial tolerance to antibiotics due to co-regulation of resistance genes (Baker-Austin et al., 2006). Although determining the genetic mechanisms responsible for the co-resistance is still unclear, experiments using molecular genetics may help to provide details (Zhang et al., 2009).

It has been suggested that resistant bacteria can be selected in aquaculture by the deposition of heavy metal ions such as Cu, Zn, and Hg in sediments (Burridge et al., 2010). Baker-Austin et al. (2006) reported that tolerance levels to metals such as Cd and nickel (Ni) and antibiotics like ampicillin and tetracycline were

the highest for bacteria found in sediment comparing to bacteria in biofilms of the water column (Baker-Austin et al., 2006).

Evaluation of the concentrations and accumulation of heavy metals in an aquaponic system may help to determine the potential contamination in water, animals, or plants. The goals of this experiment were to determine the effect of the potential accumulation of metalloid (As), and some heavy metals Cd, Pb, and Hg) in an aquaponic system, and to estimate bacterial resistance to two commonly-prescribed antibiotics (ampicillin and tetracycline). Evaluation of the BAR in the aquaponics system may help in assessing the potential hazards to the human health that may arise from using heavy metal-contaminated water in an aquaponic system.

Co-selection of heavy metals and antibiotics

Antimicrobials contribute to antibiotic resistance when they exert selective pressure via killing susceptible bacteria, allowing antibiotic-resistant bacteria to survive and grow. In general, bacteria can be resistant to antimicrobial agents such as antibiotics and heavy metals. For instance, various studies have shown that the toxicity of heavy metals can be resisted by certain microorganisms, even at high concentrations through the gaining of specific resistance mechanisms such as uptake mechanisms and efflux, and extracellular precipitation (Kamika and Momba, 2013).

Co-selection is the natural selection associated with two or more genes that are expressed when the organism is exposed to a single selective factor. Because genes encoding for resistance to heavy metals and antibiotics can be located on

the same mobile genetic element (such as a plasmid) (Baker-Austin et al., 2006), it has been recognized for a long time that a co-resistance can occur. For example, long exposure to a heavy metal can, in effect, promote the resistance development and "turn on" genes encoding for antibiotic resistance of the surrounding bacterial community.

Cross-resistance can also occur when different antimicrobial agents (two or more) attack the same bacterial target. For example, antimicrobials and heavy metals may target the bacterial cell membrane. In this case, developed resistance to the antimicrobial may also provide resistance to the heavy metal because the same gene regulates them, and gene expression is cross-regulated by the same factor (Di Cesare et al., 2016; Chapman, 2003).

As a summary, bacterial mechanisms for resistance to different chemical stressors can be either co-resistance or cross-resistance; co-resistance results from different resistance genes present on the same transposable element. Cross-resistance occurs when there is only one resistance gene expressing resistance to multiple stressors (for example, to antibiotics and metals). In effect, metal contamination contributes to selection pressure in environmental bacteria, which potentially contributes to the spread of antibiotic resistance (Baker-Austin et al., 2006). Summers et al. (1993) evaluated the effects of Hg released from dental fillings on bacterial resistance to Hg and antibiotics in oral and intestinal flora. The authors reported that, despite no recent exposure to antibiotics, levels of bacterial resistance to Hg and several antibiotics (ampicillin, tetracycline, kanamycin, streptomycin, and chloramphenicol) were very high, suggesting that

these resistances are genetically linked. Similar findings were reported by Ghosh et al. (2000), who found that *Salmonella* strains were resistant to some metals (As, chromium (Cr), Cd, and Hg) and were also resistant to ampicillin. When the plasmids that encoded resistance were transferred into a non-resistant strain of resistance to the metals and antibiotics was transferred to the new bacterium. Finally, Berg et al. (2010) studied the effect of Cu pollution in soil, revealing that the contaminated soil samples selected not only for Cu-resistant bacteria, but also co-selected for resistance to several antibiotics, including tetracycline, olaquindox, nalidixic acid, chloramphenicol, and ampicillin.

Antibiotic studied

Antibiotics have been used on a large-scale since they were first discovered as effective therapeutic agents against infectious diseases. However, the rapid worldwide spread of antibiotic resistance threatens their continued use (MacGowan and Macnaughton, 2013; Wright and Poinar, 2012); as soon as the first antibiotics appeared (penicillin and streptomycin), the problem of bacterial resistance emerged. Resistance is now a serious public health issue worldwide and, with pharmaceutical research focusing less and less on development of new antibiotics (Wright and Poinar, 2012), the problem will most probably worsen over time.

This study examined the co-selection of resistance to two commonly prescribed antibiotics (ampicillin and tetracycline) and heavy metals (As, Cd, Hg, and Pb) in a recirculating aquaponics system.

Ampicillin

Ampicillin, a beta-lactam antibiotic (Munita and Arias, 2016; Castle, 2007), is a semisynthetic penicillin derivative medical drug used for treating urinary tract infections, some respiratory infections, acute bacterial cystitis, and skin infections ("Ampicillin", 2018; Castle, 2007). It is effective against a wide range of bacteria, including some Gram-negative organisms, Gram-negative anaerobic organisms, and Gram-positive anaerobic organisms (Castle, 2007).

Ampicillin targets the bacteria cell wall by binding to the penicillin-binding proteins in the plasma membrane, which inhibits bacterial cell wall biosynthesis, resulting in the death of the cell (Castle, 2007). Potential side effects of ampicillin are skin rashes, gastrointestinal complaints and (rarely) anaphylaxis, a severe, potentially life-threatening allergic reaction ("Ampicillin", 2018; Castle, 2007).

Since its development, ampicillin has been used to overcome the penicillin resistance of bacteria such as *Staphylococcus aureus*. Ampicillin resistance has been widely reported in many bacteria. For instance, resistance levels in *Enterococcus faecium* have increased up to 80% in about 22 years since it was detected in the U.S. (Zhang et al., 2012). Ampicillin resistance spreads through genes which encode for penicillinase, an enzyme produced by particular bacteria to inactivate penicillin. In addition, plasmid-encoded β-lactamases of some Gramnegative bacteria can also confer resistance to ampicillin (Munita and Arias, 2016).

Co-selection of bacterial resistance to heavy metals and ampicillin has been a worldwide concern. Rajbanshi et al. (2008) examined bioremediation of heavy

metals in the wastewater of a sewage treatment plant and reported high resistance to heavy metals such as Cr, Cd, Ni, and cobalt (Co). Metal-resistant bacteria also showed high resistance to antibiotics: 10% of metal-resistant bacteria also showed resistance to a single antibiotic, while 90% were resistant to multiple antibiotics, including ampicillin (Rajbanshi, 2008). In a related study, Cu has been shown to promote bacterial resistance to several antibiotics including ampicillin (Berg et al., 2010).

Tetracycline

Tetracyclines are a class of antibiotics that have the same structure, but they differ by the presence or absence functional groups such as methyl and hydroxyl. Tetracyclines are low cost antibiotic with high antimicrobial activity (a broad-spectrum antibiotic) used to control both Gram-positive and Gram-negative bacteria (Cao et al., 2018). They can be isolated naturally or semi-synthetically from various bacteria of *Streptomyces* species or their compounds (”Tetracycline”, 2018). They can used for treating respiratory and urinary tract infections, prostatitis, cholera, and brucellosis. Tetracyclines can target bacterial protein synthesis and may influence the cytoplasmic membrane of the bacterial cell which leads to the leakage of vital constituents from the cell, causing cell death (“Tetracycline”, 2018; Scholar, 2007).

The European Union and United States Environmental Protection Agency list tetracyclines as emerging concern contaminants because they are increasingly present in the aquatic environment such as drinking water. It has a long half-life period in the environment due to their resistance to biodegradation (Fernandez et

al., 2018) which leads to accumulation in the environment (Cao et al., 2018). Also, most of their derivatives are not fully metabolized when ingested in humans and animals. As a result, they can be released in waters through urine and feces (Fernandez et al., 2018).

The broad-spectrum effectiveness of antibiotics such as tetracycline has led to the misuse of this antibiotic group in human and animals. High concentrations of tetracyclines have been detected in wastewater near animal farms (Burridge et al., 2010). Point sources of contamination also include effluent from pharmaceutical manufacturing facilities (Burridge et al. 2010). As a result, tetracycline can enter the aquatic environment, leading to pollution of surface waters or groundwater via soil leaching (Fernandez et al., 2018).

The pollution of water sources with tetracycline can lead to the dissemination of resistance genes in the environment. These genes can play a significant role in the distribution and dissemination of tetracycline resistance (Huang et al., 2016). For example, fish and human pathogens can share the genetic determinants of resistance to tetracycline (Burridge et al., 2010).

The co-selection process of tetracycline and heavy metal resistance has been widely studied. For example, Rasmussen and Sorensen. (1998) Evaluated the resistance of bacterial communities to heavy metals (including Hg) and some antibiotics (including tetracycline) in bacteria from sediments of a Hg-contaminated site, and reported that the frequency of bacteria resistant bacteria to Hg and tetracycline were higher in the polluted site, compared to unpolluted sediments.

Materials and methods

Bacteriological tests were conducted to determine the antibiotic resistance of bacteria to tetracycline and ampicillin using the plating method. The experiment was conducted in aquaponics systems (described in detail in Appendix 1) at the University of Arizona campus for 35 days in February and April 2018. In addition to the control system (un-spiked with metals), the waters of the treatment system were spiked with three heavy metals and one metalloid (Cd, Hg, Pb, and As) (HMs). The control and the treatment system included three replicates. HMs were spiked into the treatment systems at specific concentrations: As, Cd, Hg, and Pb; spiked at 20%, 15%, 1.5%, and 1.0%, respectively, of the Maximum Contamination Levels (MCL) of the U.S Standards for drinking water (EPA, 2018). Each replicate was stocked with 25 fingerling tilapia fish (*Oreochromis niloticus*) (25–50 g weight; Desert Springs Tilapia, Dateland, AZ), U.S. and six butterhead lettuce (*Lactuca sativa*) (seeds purchased from Johnny's Selected Seed, Winslow, ME). The hydroponic system used in this experiment was a deep water culture (DWC). A commercial fish food was used (AquaXcel Starter 5014 0.8 mm Diet, Cargill Animal Nutrition, Minneapolis, MN) with 50% minimum crude protein, 14% minimum crude fat, 2% maximum crude fiber, and 1% minimum phosphorus.

Sampling

Water samples collected weekly from all treatment and control systems starting from Day 1 (week one) until Day 35, the last week of the experiment (week 6). In the first day of the experiment and before spiking the HMs, 10 ml of

water sample collected from each replicate directly from the reservoir in (15 ml) sterile and pre-labeled plastic tubes. The tubes washed with the reservoir water two times and filled on the third time. The samples were then transferred within two hours to the microbiology lab in a cooler with gel packs (for maintaining the temperature).

Preparation

Culturing samples

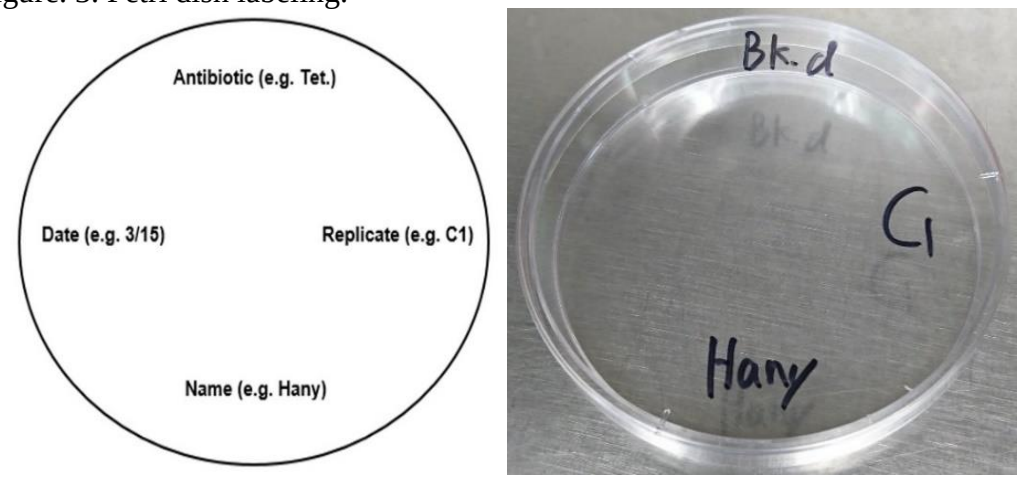
In the laboratory, all preparation of culturing plates occurred under a biosafety cabinet with HEPA filtered air to protect the samples from contamination and to protect laboratory workers from exposure to samples. For the microbiological culturing of the water samples, diluted (x0.1 deionized water) and non-diluted samples were prepared and plated on six Petri dishes; three diluted and three undiluted. Control plates contained only Mueller-Hinton agar (Remel, Lenexa, KS), while other plates contained agar supplemented with ampicillin (50 mg/ml stock solution) (Teknova Inc., CA, USA) at targeted concentration (32 µg/mL) which represent the threshold level that isolated bacteria can be a susceptible or exhibit a resistance to the ampicillin, or agar supplemented with tetracycline (50 mg/ml stock solution) (Amresco Inc., OH, USA) at targeted concentration (16 µg/mL) also represent the threshold level that isolated bacteria can be a susceptible or exhibit a resistance to the tetracycline. The target breakpoints followed guidelines released by the Clinical and Laboratory Standards Institute (CLSI, 2019). [Note: Additional information on antibiotic dilution can be found on Page 195].

In total, 36 Petri dishes were prepared on each sampling date for the six replicate water samples (18 diluted, and 18 undiluted). Plates were labeled as indicated in Figure 3. During plating, 100 µl of each water sample was transferred into the plates, then the sample was spread evenly across the plate using a disinfected cell spreader.

After 24 hours of incubation (at 37° C), colonies were counted on all plates. Data was collected on the percentage of total cultivable bacteria that are resistant to each of the target antibiotics.

Additional detailed information on all laboratory procedures can be found in Appendix A.

Figure. 3. Petri dish labeling.



Safety precautions

Safety precautions were used during all work in the lab. Personal Protective Equipment (PPE) was worn all times; this included safety glasses, a breathing mask, a laboratory coat, appropriate shoes, and double gloves. Also, insulated gloves were used for handling hot objects like flasks and hot surfaces. Other

precautions included proper cleanup of spilled chemicals or hazardous solutions, cleaning of all surfaces after finishing each step, and cleaning of the biosafety cabinet at the end of work. Additional safety precautions included washing hands before leaving the laboratory and avoiding bringing any food or drink into the lab. All work was performed under the direct supervision of the Biosafety Approval Safety Coordinator of the lab.

Results

The following charts and tables display the bacterial sensitivity and their resistance to ampicillin and tetracycline.

Table. 1. Total bacteria count of the antibiotics for the control and the treatment of the heavy metals (diluted samples) (B= blank, Amp=ampicillin, and Tet=Tetracycline).

Treatment/replicate	Antibiotic	Bacteria count					
		Week1	Week2	Week3	Week4	Week5	Week6
Control 1	B.	1460	1444	37	19	26	28
	Tet.	0	0	0	1	0	0
	Amp.	1164	1240	15	13	8	6
Control 2	BK.	1496	752	11	29	13	10
	Tet.	0	0	0	0	0	0
	Amp.	1168	840	15	5	1	6
Control 3	B.	1004	177	11	21	19	103
	Tet.	0	0	0	0	0	0
	Amp.	752	41	4	12	2	124
Treatment 1	B.	628	292	21	24	23	11
	Tet.	0	9	3	3	0	0
	Amp.	296	133	12	2	8	1

	B.	636	300	5	14	109	13
Treatment 2	Tet.	0	6	2	3	1	0
	Amp.	480	175	5	2	69	1
	B.	572	119	24	24	18	5
Treatment 3	Tet.	0	5	1	1	0	0
	Amp.	276	94	11	6	5	0

Ampicillin

Table 2 shows the bacterial resistance to ampicillin of the control and the treatment water samples (diluted). All data is presented as a ratio of bacterial colonies on the ampicillin plates vs. bacterial colonies on the control (no antibiotic) plates. Thus, any number less than 1.0 indicates that less bacteria grew when exposed to the antibiotic. As Table 2 shows, bacterial growth gradually decreased in control and in treatment samples with exception of the control samples (Replicate 3) where bacterial growth increased during week 5.

Over the length of the experiment, bacteria presented resistance to the ampicillin in both the metal-treated water samples and the control samples, although ampicillin was effective in reducing overall bacterial growth (Table 2), growth persisted on all plates during all sampling events, with the exception of the final sample collected from the third replicate of the metal-treated aquaponics tank. When compared to the control samples, the results of the metal-treated samples did not indicate that the HMs promote antibiotic resistance of the bacteria over a six-week period. However, HMs affected the bacterial growth negatively in all samples with an exception for week five where more bacteria grew in the

treated samples compared to the control. In the final week of sample collection, BAR in the control samples increased again, but it decreased in the treated samples. Over the length of the study, there was no difference between bacterial growth in the samples of metal-treated or control tanks ($P > 0.05$) (Fig. 4; Table 3).

Table. 2. Bacterial resistance to ampicillin for the control and the heavy metals-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the total bacterial counts on each plate, as a ratio to the total bacterial counts on plates without added the antibiotic.

Week	1	2	3	4	5	6
Control 1	0.80	1.08	0.41	0.68	0.31	0.21
Control 2	0.78	1.12	1.36	0.17	0.08	0.60
Control 3	0.75	0.23	0.36	0.57	0.11	1.20
Treatment 1	0.47	0.46	0.57	0.08	0.35	0.09
Treatment 2	0.75	0.58	1.00	0.14	0.63	0.08
Treatment 3	0.48	0.79	0.46	0.25	0.28	0.00

Figure. 4. The average of the bacterial resistance to ampicillin for the control and the heavy metal-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the total bacterial counts on each plate, as a ratio to the total bacterial counts on plates without added antibiotic. The error bars represent the standard deviation (S.d).

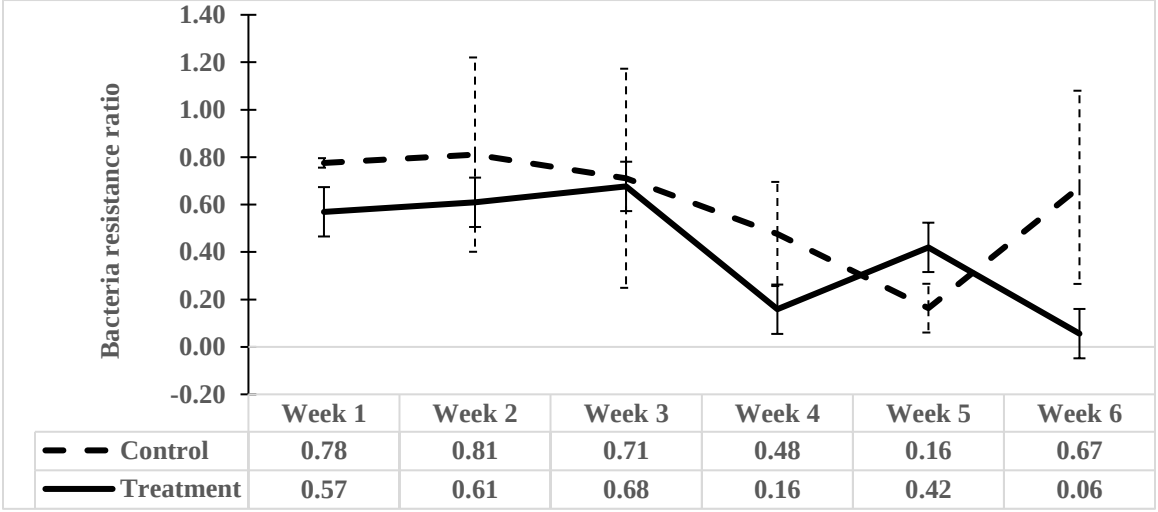


Table. 3. The average of the bacterial resistance to ampicillin for the control and the heavy metal-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the average total bacterial counts on the heavy metal-treated plates (three plates), as a ratio to the total bacterial counts on the control plates (three plates) without added the antibiotic. The average means± and the standard deviation (S.d) of the control and the heavy metals-treated data included in addition to the P-value of the means is included

	Control		**Treatment**		**P-value**
	Mean±	**Standard deviation**	**Mean±**	**Standard deviation**	
Week 1	0.78	0.02	0.57	0.13	0.14
Week 2	0.81	0.41	0.61	0.14	
Week 3	0.71	0.46	0.68	0.23	
Week 4	0.48	0.22	0.16	0.07	
Week 5	0.16	0.10	0.42	0.15	
Week 6	0.67	0.41	0.06	0.04	

Tetracycline

The bacterial resistance to tetracycline in 0.1X diluted water samples for both the control and the treatment is shown in the (Table 4). The BAR was absent in all control samples except for week four when a single colony was observed. In the metal-treated tanks, BAR progressively increased from the first week to the third week; thereafter, it decreased until the end of the experiment (Table 4; Fig. 5). The observed resistance to tetracycline was significantly higher in the metal-treated samples compared to the control over the length of the study ($P < 0.05$) (Table 5).

Table. 4. Bacterial resistance to tetracycline for the control and the heavy metals-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the total bacterial counts on each plate, as a ratio to the total bacterial counts on plates without added the antibiotic.

Week	1	2	3	4	5	6
Control 1	0.00	0.00	0.00	0.05	0.00	0.00
Control 2	0.00	0.00	0.00	0.00	0.00	0.00
Control 3	0.00	0.00	0.00	0.00	0.00	0.00
Treatment 1	0.00	0.03	0.14	0.13	0.00	0.00
Treatment 2	0.00	0.02	0.40	0.21	0.01	0.00
Treatment 3	0.00	0.04	0.04	0.04	0.00	0.00

Figure. 5. The average of the bacterial resistance to tetracycline for the control and the heavy metal-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the total bacterial counts on each plate, as a ratio to the total bacterial counts on plates without added antibiotic. The error bars represent the standard deviation (S.d).

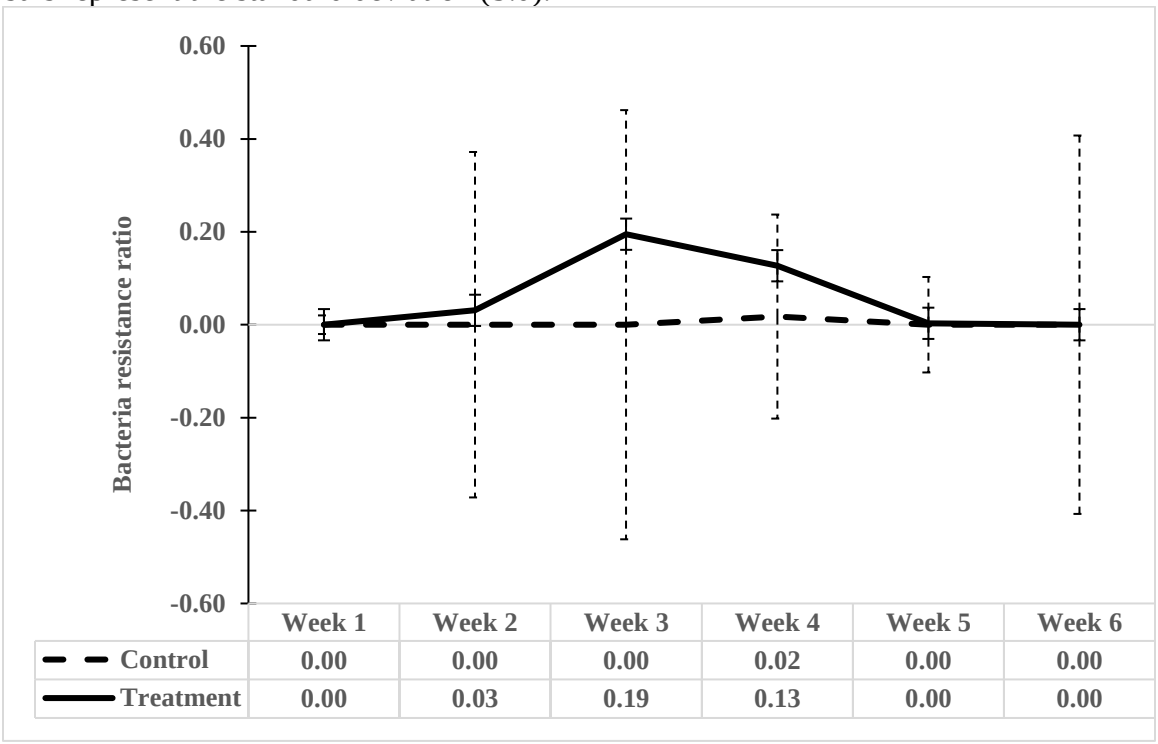


Table. 5. The average of the bacterial resistance to ampicillin for the control and the heavy metal-treated of aquaponics water samples. Data presented is for diluted (0.1X) samples only. Numbers represent the average total bacterial counts on the heavy metal-treated plates (three plates), as a ratio to the total bacterial counts on the control plates (three plates) without added the antibiotic. The average means± and the standard deviation (S.d) of the control and the heavy metals-treated data included in addition to the P-value of the means is included

	Control		Treatment		P-value
	Mean±	**Standard deviation**	**Mean±**	**Standard deviation**	
Week 1	0.00	0.00	0.00	0.00	0.04
Week 2	0.00	0.00	0.03	0.01	
Week 3	0.00	0.00	0.19	0.15	
Week 4	0.02	0.02	0.13	0.07	
Week 5	0.00	0.00	0.00	0.00	
Week 6	0.00	0.00	0.00	0.00	

Discussion

In general, the result of the BAR studies show that the mixed HMs inoculated into the treatment did not promote BAR to ampicillin. Though the mixed HMs did promote resistance to tetracycline, this effect appeared to be only temporary, as resistance was not found in the final two sampling periods.

The ampicillin bacteriology results of the control water samples (first replicates) and the treated water samples (second replicates) may indicate that the BAR to ampicillin could have already been established in the source water before starting the experiment. However, by the final week of sampling, BAR to ampicillin decreased in the metal-treated tanks, while control tanks showed generally higher bacterial counts. It is difficult to come to conclusions from this, because data variability was very high. For example, in the final week of sampling, bacterial counts were 6, 6, and 124 colonies for the control replicates 1, 2, and 3, respectively (Table 1). Similarly, the second replicate of metal-treated

group on the fifth week of sampling showed unusual bacteria numbers (8, 69, and 5 colonies for the replicates 1, 2, and 3, respectively) (Table 1). The elevated bacterial counts in a single replicate is unclear. Though stringent methods were used to prevent contamination, there is a possibility that an individual sample could have been contaminated during collection or transport. It is also possible that the elevated numbers for replicate 2 could be related to leakage that was observed from this tank (dilution decreased). The water level for this replicate was the lowest compared to the volume of the water in all replicates. Substantial leakage could contribute to more concentrated bacteria in the water sample. Overall, the statistical analysis revealed no difference in BAR to ampicillin between the control and the treated tanks ($P > 0.05$) (Table 3).

The results of the bacterial growth for the tetracycline did not indicate that HMs promoted the BAR in the long run; though statistical analysis indicated that metal-treated samples had higher BAR, resistance in all samples (treated and control) fell to near zero during the final two weeks of sampling. Control samples showed a high susceptibility to tetracycline, as few-to-no bacteria were observed in any plates (Table 4).

The results of this work indicate that the BAR could not be elevated, or could not persist to the end of the experiment, in metal-treated aquaponics tanks, suggesting that, in general, the targeted concentrations of the HMs did not promote the co-selection pressure to the tested antibiotics. However, as mentioned above, there was a notable elevation in BAR in HM-treated tanks for three weeks in the middle of the experiment. This elevation corresponded to increased

accumulation of HMs in the water (notably As and Cd), but this correlation did not persist after week 5, suggesting that water conditions had become intolerable for the tetracycline-resistant bacteria. Potential factors include interaction of the exposure time of the HMs on the bacteria life, and the pH level of the water and its effect on the antibiotic activity.

Due to competition for binding sites in cellular proteins, even essential metals such as Zn can be toxic for the bacteria if they accumulate in excess in the bacterial cell (Lim et al., 2013). The toxicity of any heavy metal to an organism depends on the HM concentration and chemical form (Bosch et al., 2016). Some bacteria can tolerate the toxicity of heavy metals because their genome contains resistance genes that encode for processes that excrete metals (Silver, 1996). However, if the bacteria cannot excrete the accumulated toxic ions from the cell over time, they die.

Because bacteria have the ability to transfer resistance genes from one isolate to the next, it may be expected that over time, bacteria would exhibit higher levels of resistance to metals when exposed to elevated concentrations. Furthermore, since the mode of resistance to heavy metals and antibiotics can be the same, it may be expected that bacteria in heavy-metal contaminated sites may become increasingly resistant to antibiotics. Zhang et al. (2018) examined samples collected from long-term Cu-contaminated sites. They reported that bacterial resistance to several human antibiotics increased after being exposed to concentrations of Cu between 10 and 100 mg/L, which is about 10-100 times greater than the MCL standard by the (EPA, 2018).

Chen et al (2015) investigated the effects of several heavy metals (As, Cu, and Zn) in selecting for antibiotic resistance of a Gram-negative bacterium (*Enterobacteriaceae* family; LSJC7). The results showed that the bacterium expressed multi-resistance to the metals as well as to tetracycline, and they found that the presence of As promoted the tetracycline resistance of the bacteria. However, the growth of LSJC7 decreased with increasing concentrations of the metals. However, when they examined these responses in a different bacterium, *Pseudomonas oryzihabitans*, resistance to tetracycline was not promoted in the presence of As; moreover, tetracycline resistance overall decreased with increasing metal concentrations. These results indicate that co-selection for metal and antibiotic resistance may show different patterns in different bacteria. In our study, bacterial resistance to tetracycline resistant increased over the time, corresponding to the increased concentrations of HMs in the system till to the end of the week five. The reduction in resistance in the last two weeks may result from the combined influences of the different HMs with the tetracycline, particularly As which reached high levels in the water in the replicates 1 and 3 while the second replicate exceeded the MCL. However, include the use of molecular analyses, which has not been done in the current study, is important to provide a better understanding of this case. Also, applying molecular test can help to identify and quantify resistance genes and studying the individual lethal concentration of each metal.

On the other hand, water physicochemical factors such as pH, can affect the influence of antibiotics on bacteria (Smith et al., 1994; Burridge et al., 2010;

McArdle et al., 2018; Percival et al., 2014). Antibiotics showing pronounced effects include tetracycline (Huang et al., 2016; Bartek et al., 2016; Yang et al., 2014; Al-Mariri and Safi, 2013). Huang et al. (2016) proposed that different pH levels might change the natural biofilm structure of tetracycline-resistant bacterial communities, leaving them exposed and enhancing the activity of the antibiotic.

Percival et al. (2014) proposed that pH effects on the activity of antibiotics may not be related to increases or decreases in bacterial tolerance to antibiotics, but that pH variations can control the binding of certain antibiotics to their cellular target sites. In their study where they evaluated the effects of pH on healing of wounds as well as on the antimicrobial efficacy. They suggested that gentamicin is less able to transport into the cell under acidic conditions due to the large ionization of the antibiotic at a more acidic pH compared with neutral pH conditions; however, they stated that due to alterations of the metabolic state of bacteria, tolerance to antimicrobials at certain pH ranges may increase (Percival et al., 2014). Al-Mariri and Safi evaluated the activities of some antibiotics, including tetracycline, at different pH levels (5 and 7) against *Brucella melitensis*. The study found that the tetracycline was significantly ($P < 0.01$) more effective at pH 7 compared to pH 5 (Al-Mariri and Safi, 2013).

Thus, pH external to the bacteria is an important controlling factor, but internal pH is also critical. Bartek et al. (2016) suggested that internal pH homeostasis could be a mechanism whereby bacteria can combat antibiotics. For example, they found that *Mycobacterium smegmatis* and other bacterial pathogens were able to impede the activity of several antibiotics by maintaining their

intracellular pH homeostasis. In addition, they suggested that antibiotics may be able to kill bacteria by inhibiting the ability to maintain their intracellular pH homeostasis. Antibiotics can do this by inhibiting the entry of protons into the cells by inhibiting the cellular proton pumping mechanism, thus leading to alkalinized intracellular pH and cell death.

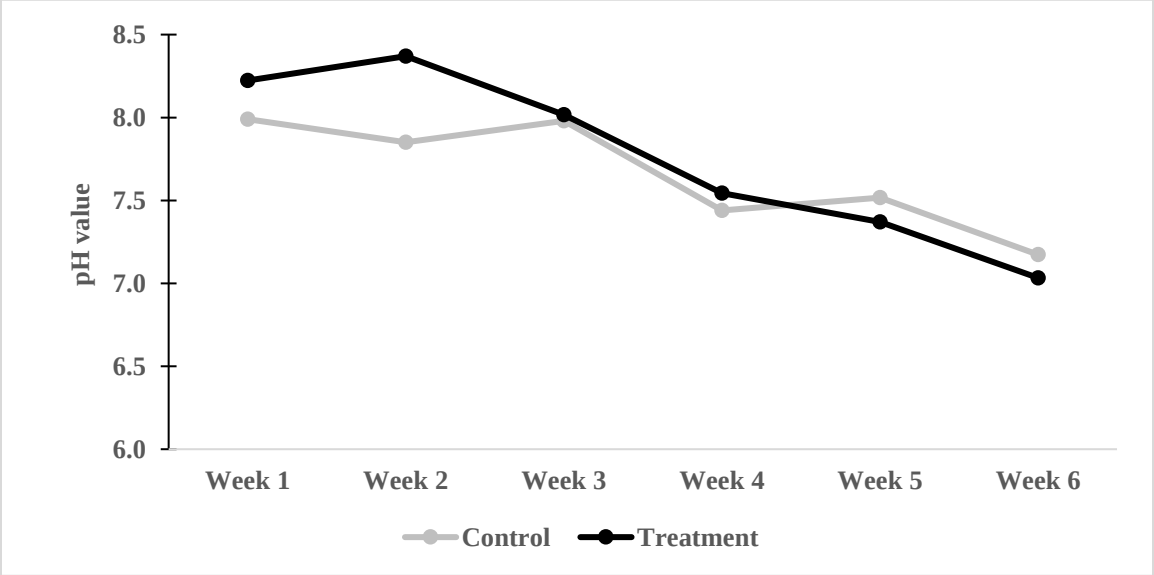
Huang and his colleagues (2016) studied the potential effect of different pH levels on the distribution of tetracycline-resistance genes in anaerobic treatment of waste sludge. They found that the number of tetracycline genes was promoted at low pH level (acidic environment) but restricted in alkaline levels. However, the study also revealed that two tetracycline-resistant bacterial groups were affected differently by pH; *Proteobacteria* were predominant at lower pH levels, while *Firmicutes* thrived at higher levels of pH (Huang et al., 2016).

Yang et al. (2014) reported that pH plays a significant role in the inhibitory activity of antibiotics against bacteria. Activity of antibiotics (including tetracycline and β lactam antibiotics) improved with decreasing pH in a range of pH 5 to pH 8. Percival et al. (2014) reported that pH has an effect on the minimal inhibitory concentrations (MIC) of bacteria. It has been found that MIC at high pH was lower of certain bacteria to antibiotics compared with neutral or low pH. For instance, the MIC of the beta-lactams bacteria decreased in low pH levels compared to neutral pH. On the other side, other studies also showed opposite results where low pH levels lead to higher MICs for other ranges of antibiotics.

Over 35 days of operation of our aquaponics systems, water pH levels decreased in both the control and HM-treated systems; it decreased in the

treatment from 8.2 in the first week to 7.5 in the 4th week and finally reached to 7.0 (Fig. 6). It's not clear whether decreasing the pH from mid-alkaline to neutral enhanced the activity of ampicillin and tetracycline or not; this could have been masked by the complexity of the bacteria in the system. However, further experiments as well as bacterial molecular analyses are necessary to provide clearer evidence to explain the behavior and the efficiency of these antibiotics in this water condition.

Figure. 6. Weekly average pH values (control and treatment) during the experiment period.



In general, it is noticeable how different the results are from previous studies in determining the effect of pH on the antibiotics efficiency on several species of bacteria; the results of these studies were varied. Also, due to the relatively low number of data points we had in our experiment, it is difficult to interpret the relationship of the pH with the antibiotic efficiency on bacteria in the water.

In conclusion, the resistance of the bacteria decreased gradually, in general, by the end of the experiment. This may refer to the elevated direct toxic effect of the HMs on the bacteria viability; the bacteria may have been affected after the third week by the contacted toxic of the HMs elevated concentration in the water, and/or may due to the slight decrease of the pH (from 8 to 7).

Future work

For clarification of the results, future studies may need to involve several aspects such as sampling of the sediments, use molecular tests for studying the interaction effect of other metals on the bacteria, continue the experiment for longer periods and may be with higher metal concentrations, and evaluate the bacterial resistance to additional antibiotics.

Sampling sediments of the systems and bacteria samples from the sediments should be considered to improve the sample collecting process in future experiments. As mentioned above, according to Baker-Austin et al. (2006), benthic bacteria/bacteria living in sediments might be sources of metal and antibiotic resistance genes in stream ecosystems. Dependent on the gradient of metal contamination for different stream microhabitats, tolerance levels to metals and antibiotics (Cd, Ni, ampicillin, and tetracycline) were the highest for bacteria that found in sediment comparing to bacteria in biofilms of the water column. We should take into the consideration that binding antibiotic to organic matter in the sediments may decrease the antibiotic activity due to high pH and salt concentrations (Burridge et al., 2010). Applying molecular tests in the future work

may help to determine which metal that bacteria were able to resist or not. Running the experiment for longer period may be reasonable to obtain more objective results to study the effect of the accumulation of the metal/metals on the BAR. Finally, in addition to ampicillin and tetracycline, other antibiotics can be tested as well for the bacterial resistance.

Conclusion

As a conclusion, (Fig. 7, 8, 9, and 10) showed the elevated concentration of the toxic HMs in the water over the time of the experiment. Two main factors may be involved with the result. The potential increase of the antibiotics activity (ampicillin and tetracycline) at pH=7, and the elevated concentrations of the toxic HMs after the fourth week, particularly As. These factors may have enhanced each other and worked in association to overcome the tolerance of the resistance bacteria by the end of week 4.

Annex 2

Labeling plates

While the media were cooling, Petri dishes were prepared for pouring the media (agar). The plates were labeled with the replicate codes (C1, C2, C3, T1, T2, and T3) where C represent the control, and T represents the contaminated water (that were spiked with HMs), as well as labeled for the antibiotic treatments and the control as the following codes. Ampicillin, tetracycline, and the control (blank) were coded as Amp, Tet, and B, respectively (Fig. 3).

Medium preparation

Mueller-Hinton Agar solution was used for the culture medium of the treatments (Ampicillin, tetracycline, and the control). After autoclaved the solutions, the flasks of the solution were transferred to a water bath at 55 °C for about 15 min until cooled. After cooling the media, flasks were transferred into a safety cabinet for the antibiotics inoculation and then for pouring the agar media. The plates were used directly for culturing the bacteria or stored in the refrigerator to use later in a week at maximum. In cases using the stored ones, the plates were warmed in the incubator for 45-60 min before starting the culturing test.

Calculations for dilution of antibiotics

Ampicillin

The ampicillin concentration in the stock solution from the manufacturer was 50 mg/ml. The target concentration was 16 µg/ml = 0.016 mg/ml. The amount of the stock solution containing the target amount of ampicillin was calculated as:

$$\frac{0.016\ \text{mg/ml}}{50\ \text{mg/ml}} = 0.00032\ \text{ml} = 0.32\ \mu\text{l}$$

Thus, 0.32 µl of stock solution had to be added to each mL of diluent to achieve the desired concentration. Because 300 mL of Mueller-Hinton agar was prepared:

0.32 µl x 300 = 96 µl

Therefore, 96 µl of ampicillin was added into the flask containing 300 mL of Mueller-Hinton agar.

Tetracycline

The tetracycline concentration in the stock solution from the manufacturer was 50 mg/ml. The target concentration was 32 µg/ml = 0.032 mg/ml. The amount of the stock solution containing the target amount of tetracycline was calculated as:

$$\frac{0.032\ \text{mg/ml}}{50\ \text{mg/ml}} = 0.00064\ \text{ml} = 0.64\ \mu\text{l}$$

Thus, 0.64 µl of stock solution had to be added to each mL of diluent to achieve the desired concentration. Because 300 mL of Mueller-Hinton agar was prepared:

0.64 µl x 300 ml = 192 µl

Therefore, 192 µl of tetracycline was added into the flask containing 300 mL of Mueller-Hinton agar.

Protocols

The following is a detailed description of laboratory procedures used in this study.

Media preparation

1. Turn on the water-bath at 55 °C.
2. Prepare three sterile 1-liter glass flasks for the Mueller-Hinton Agar solution (three flasks 300 ml of agar in each). Flasks will contain agar with antibiotics (one ampicillin flask, one tetracycline flask), and one will contain agar for control agar (no added antibiotics).
3. Each flask must be labeled with a code that represents the antibiotic treatment or the control using: *Amp.* For Ampicillin, *Tet.* For tetracycline, or *B.* for a blank (or control) sample which will not be inoculated with an antibiotic. Labeling the flasks is done with a Sharpie marker to ensure permanency of writing.
4. Add 300 ml of the deionized water to each flask.
5. Use a weighing boat and a micro- scale to weigh the amount of Mueller-Hinton powder needed (38g /1 liter of deionized water = 11.4 g/300 ml) for each flask.
6. Add the agar powder to each flask.
7. Add magnetic sterile stir bar, cap loosely, and put the flask on the hot plate at 100 °C with 3° stir. Remove the flask when boiling start.
8. Transfer the flasks to a water-bath at 55 °C then cap the flask tightly. We do not need the agar to cold quickly and solidify before do the autoclaving step so we keep it here until finish the other flasks.
9. Repeat the steps 3-8 for the other two flasks.
10. Loosen the caps of all three flasks and place them into the autoclave.

11. Autoclave the flasks at 121°C with 15 lbs pressure for about 50 min hour to sterilize the agar.
12. From the autoclave, transfer the flasks to the water bath at 55 °C for about 15 min until they are cool. Higher temperature will degrade the antibiotics that will be added later.
13. While waiting for the media to cool, label 36 sterile Petri dishes. All plates were labeled with a code corresponding to one of the aquaponics replicates (C1, C2, C3, T1, T2, or T3) and the treatments (*Amp.*, *Tet.*, or *B*) must be coded. Thus, each replicate of the experiment has three plates; the first was used to culture a water sample on a blank (no antibiotic) plate; (as B.), the second for ampicillin, and the third plate for tetracycline (as Tet.).
14. Remove the flasks from the water bath and place them into the biosafety cabinet to inoculate the antibiotics, according to the calculations in the antibiotics inoculation protocol.
15. After the inoculation, swirl the agar to mix the antibiotics thoroughly. Open the cap of the flask with one hand and use the other hand to open the Petri dish then pour the agar medium (approximately 25 ml of for the 100 mm plates). Repeat this step until pouring the plates needed.
16. Store the plates upside down in a refrigerator until use.

Samples plating

1. The plating of the water samples for bacterial culturing is done in a biosafety cabinet.

2. Before plating, warm the refrigerated plates in an incubator at 37 °C for 45-60 minutes.
3. Prepare six sterile 15 ml tubes for use in diluting the water samples.
4. Prepare one flask with 10 ml of deionized water (DiW).
5. In the biosafety cabinet, use a sterile pipet to transfer 900 µl of the DiW to each of the sterile 15 ml tubes. Transfer 100 µl of each water sample to the tube containing the 900 µl of the DiW, for a 10-fold dilution of each water sample.
6. Inoculate the sample into the Petri plate; use a sterile pipet to collect 100 µl. This step is completed for each sample, both diluted and un-diluted.
7. After finishing the plating, let the Petri plates sit under the biosafety cabinet for 40 min to ensure attaching the water (and the bacterial cells) onto the agar.
8. Transfer the plates into the incubator at 37 °C for 24 hours. The plates must be in upside down position to avoid any condensation that may drip onto the plates and protect the bacterial communities to do not be interfered when they grow.
9. When plates are removed, count all bacterial colonies on each plate. Any plate with more than 300 bacterial colonies is noted as “too numerous to count” (TNTC).

FIGURES

Figure. 7. Arsenic concentration of the control and the treatment in the water during the experiment period.

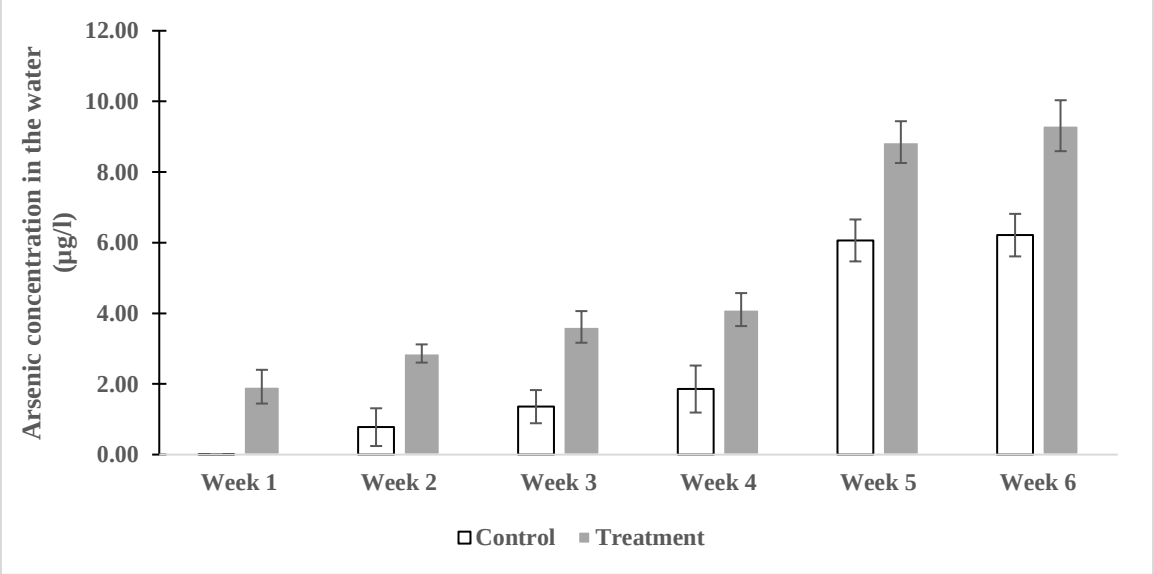


Figure. 8. Cadmium concentration of the control and the treatment in the water during the experiment period.

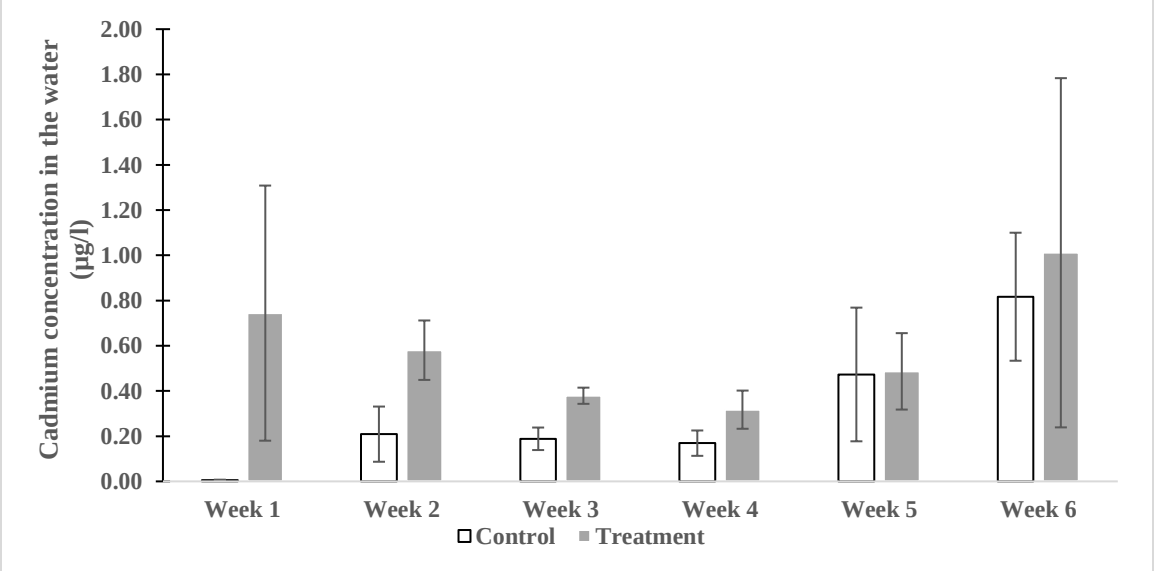


Figure. 9. Mercury concentration of the control and the treatment in the water during the experiment period.

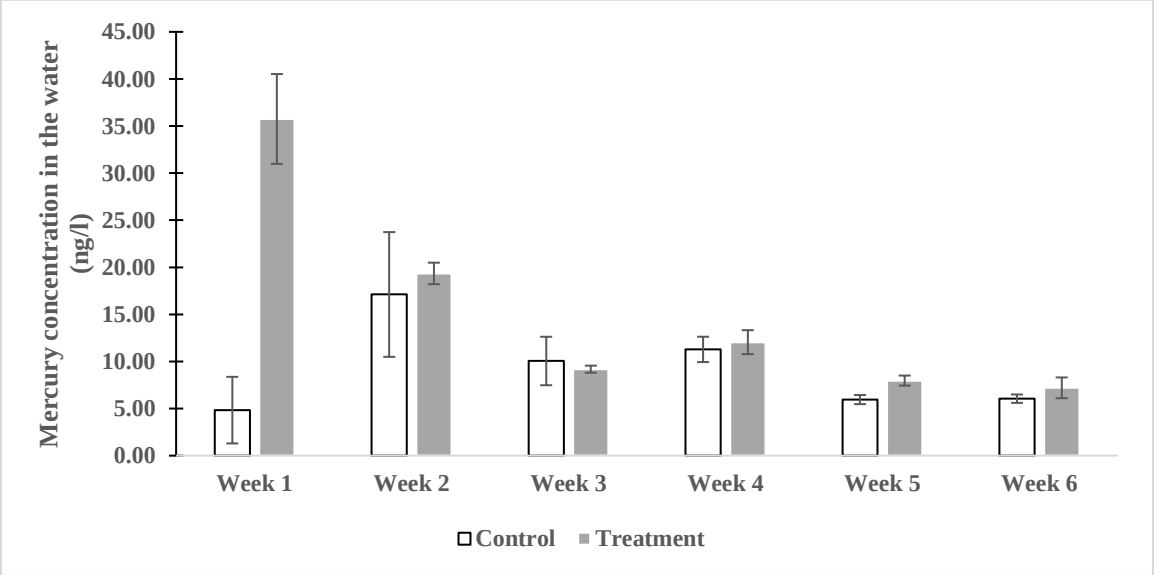
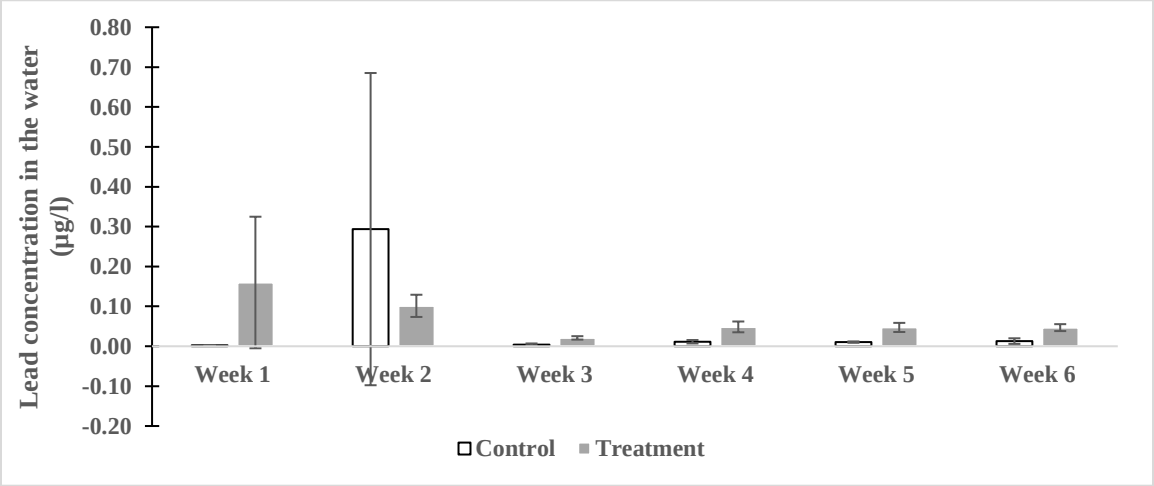


Figure. 10. Lead concentration of the control and the treatment in the water during the experiment period.



TABLES

Table. 6. Total bacteria count of the antibiotics for the control and the treatment of the heavy metals (undiluted samples) (B= blank, Amp=ampicillin, and Tet=tetracycline).

Treatment/replicate	Antibiotic treatment	Bacteria count					
		Week1	Week2	Week3	Week4	Week5	Week6
Control 1	B.	4476	1472	*	238	206	253
	Tet.	0	0	*	12	0	0
	Amp.	3488	2144	*	121	103	176
Control 2	BK.	2596	1404	*	257	95	63
	Tet.	41	11	*	3	1	0
	Amp.	1752	1564	*	25	28	44
Control 3	B.	2856	3472	*	93	49	780
	Tet.	3	14	*	0	0	0
	Amp.	2016	1336	*	43	21	392
Treatment 1	B.	1992	1688	*	85	173	28
	Tet.	0	552	*	29	0	2
	Amp.	904	1544	*	105	69	26
Treatment 2	B.	2808	1864	*	174	1344	164
	Tet.	13	32	*	8	3	10
	Amp.	1368	1936	*	92	716	34
Treatment 3	B.	2440	3008	*	142	128	97
	Tet.	4	12	*	6	0	0
	Amp.	2392	1872	*	68	88	32

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APPENDIX C - EFFECT OF SOME TOXIC HEAVY METALS/LOID ACCUMULATION ON LENGTH OF LETTUCE ROOT AND SHOOT IN A SMALL-SCALE AQUAPONIC SYSTEM

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ABSTRACT

Aquaponics combines aquaculture and hydroponics for rearing fish and plants growing in a soil-less system with recirculating water. It is a sustainable technology that holds promise to enhance global food production and save water in an efficient manner. Due to trace amounts in fish feed, and natural loss of water through evaporation and transpiration, organic and inorganic pollutants such as heavy metals (HMs) may concentrate in fish and plants. This may pose health threats to consumers when consuming contaminated food.

Effect of some toxic heavy metals and metalloid (arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)) (HMs) on the growth of lettuce was investigated by evaluating the length of the root and the shoot (edible leaves) and their masses (dry weight) in a small scale aquaponic system. Based on the maximum contamination level (MCL) set by the EPA for potable water, different levels of the HMs were inoculated in the water as a treatment. Three replicates were prepared for the treatment and the control each, and each replicate was stocked with 25 Tilapia and 6 lettuce plants. The treatment replicates received mixed concentrations of the HMs: As, Cd, Hg, and Pb at 20%, 15%, 1.5%, and 1.0% of the MCL, respectively. Root and shoot lengths of the lettuce plants were measured on the last day of the experiment to examine the effect of the HMs that had accumulated in the system by the harvest day. There were no significant differences in root or shoot lengths or biomass between the treatment and control. The results did demonstrate bio-accumulation of the HMs in the root

Introduction

Heavy metal pollution is a serious concern because of their toxicity and their mobility as ions in water (Demirbas, 2008). This concern has increased due to the need in many countries for reusing water impacted by industrial processes (Tofighy and Mohammadi, 2011) in aquaculture, hydroponics, and aquaponics. Metals ions are frequently found in the industrial wastewater that eventually ends up in potable water sources (Demirbas, 2008). Heavy metals do not degrade biologically, so they may accumulate in the environment, especially in water and thus into aquatic organisms which may pose a public health problem (Tofighy and Mohammadi, 2011).

In aquaponic systems, plants and fish are the primary components. Plants serve two goals in an aquaponic system; they grow as a source of food, and due to the symbiotic relationship between the fish and the plants in the system, they can remove toxic metabolic wastes, such as ammonia, which can be harmful to fish. Some plants function as hyperaccumulators and can be used as a biological agent to remove toxic contaminants such as heavy metals. This technology is called phytoremediation, where plants can gather pollutants from the surrounding environment such as soil, water or air (Nazir et al., 2011). 400 plant species are known to be hyper-accumulators for metals such as nickel (Ni), zinc (Zn), or cobalt (Co) capable of reaching exceptional concentrations after extracting from the soil into aboveground tissues (Kramer et al., 1997). Other edible plants such as leafy and non-leafy vegetables are also accumulators of heavy metals (Khan et al., 2015; and some metals such as lead (Pb) and cadmium (Cd) can

bioaccumulate in the edible parts of the plants (Michalska and Asp, 2001) and thus may cause health risks to consumers (Dala-Paula Et al., 2018).

Plants taking up their essential nutrients may be absorbing non-essential elements such as toxic heavy metals like (Arsenic (As), Cd, mercury (Hg), and Pb) that are not needed and may be harmful to them, even at low concentrations (Ali et al., 2013; Lima e Silva et al., 2012; Shaw et al., 2004). Even the essential metals such as (Cu, Mn, Ni, and Zn) can be toxic to the plants at elevated concentrations (Küpper et al., 2009). For example, Cu is an essential element, but it could inhibit the growth of plants leaf at elevated levels (Bouazizi et al., 2010). Arora et al. (2008) evaluated different heavy metals like iron (Fe), copper (Cu), manganese (Mn), and Zn with various levels in vegetables that were irrigated with wastewater from different sources. They found that the plants accumulated these metals in their tissues at different levels. However, the concentrations were below the recommended maximum tolerable levels of FAO (Food and Agriculture Organization), and WHO (World Health Organization) (Arora et al., 2008). The effect of Cu stress on young and expanding tissues is poorly understood. It is known that Cu can inhibit cell division, but it is essentially unknown how this effect is mediated (Khan et al., 2015; Bouazizi et al., 2010). Heavy metals such as Hg, Cd, and Pb are toxic not only for plants but also for humans and animals even at low concentrations (Gothberg et al., 2002; Barker, 1972). Due to the long biological half-life of these metals, they can accumulate in the tissues (Gothberg et al., 2002). The physiochemical characteristics of a body of water may limit the

availability of an element for plants, but it may also yield favorable conditions for uptake of a non-essential element (Gjesteland, 2013).

Plants can be classified as accumulator, hyper-accumulator, or excluders based on their abilities to accumulate heavy metals (Khan et al., 2015). According to Khan et al. (2015), each heavy metal has a different bioaccumulation rate in a plant and this accumulation also depends on the capacity of different plant species (Engin et al., 2015). In general, the bioaccumulation ratio of leafy vegetable plants are higher than non-leafy vegetables. For example, lettuce, as a green leafy vegetable, is one of the hyper-accumulator plants for heavy metals which can accumulate metals in their tissues without showing any toxicity signs (Khan et al., 2015). Cobb et al. (2000) determined uptake and distribution of metals in the edible parts of different plants (lettuce, radishes, beans, and tomatoes) in contaminated soil with metal concentrations of Pb, Cd, As, and Zn. They found different plants accumulated these metals in different levels; lettuce roots and leaves accumulated similar concentrations of the four metals which may pose risks to consumers. Several studies have focused on the bioaccumulation of heavy metals in plant and crops (Khan et al., 2015; Pinto et al., 2004; Cobb et al., 2000), including lettuce (Smical et al., 2008; Khan et al., 2008; Ahumada et al., 1999). Lettuce is one of the most widely consumed leafy vegetables in the world, and with other vegetables, are considered significant sources of heavy metals in food (Dala-Paula et al., 2018). Li et al. (2015) determined the concentration of several heavy metals (Cr, Ni, Cu, Pb, and Cd) in five types of vegetables including different lettuce varieties at two contaminated sites, and among the plants studied,

the highest concentrations of metals were found in lettuce (*Lactuca sativa* L.). Crews and Davies (1985), grew six different lettuce varieties in contaminated soils with various concentrations of metals (Cd, Chromium, Cu, Pb, and Zn) and uptake of the metals increased with the increasing concentration in the soil.

Edible plants are susceptible to damage from some metals, and they can die at levels that are not high enough to be considered as toxic levels for human consumers (EPA, 2007). Non-essential elements such as toxic HMs can compete with essential elements which can inhibit metabolic activity of plants thus reflected on their growth. Lepp (1977) demonstrated that root growth could be inhibited due to the competition between Cd with Cu, and Cd with Ni within the symplastic tissue or at the apoplast/symplast interface of the root. Metal toxicity can cause multiple effects in many physiological functions of plants (Barceló and Poschenrieder, 1990). Excess metal ions can affect enzyme activity, cause oxidative damage of membranes (Barceló and Poschenrieder, 1990), inhibit metabolism processes (Küpper et al., 2009; Barcelo and Poschenrieder, 1990), disturb hormone balance, and inhibit photosynthesis (Barcelo and Poschenrieder, 1990). Elevation of metal ion concentration can inhibit root and shoot elongation (Santala and Ryser, 2009; Cheung et al., 1989).

Karuppanapandian and Kim (2013) studied the effect of Co on Indian Mustard plant (*Brassica juncea L.*) grown in a hydroponic experiment. Excessive amounts of Co in plant tissues caused many adverse effects including severe damage to the plant cells and cell membranes, reduction of the plant biomass and its growth, inactivation enzymes, and plant death. Excessive accumulation of Cr is toxic and

reduces growth and seed germination in plants (Zeng et al., 2011). Burzynski and Klobus (2004) studied the effect of different concentrations of Cu, Cd, and Pb on photosynthesis of cucumber leaves (*Cucumis sativus L.*), in addition to decreasing plant dry mass, water percentage, chlorophyll contents, and Fe were decreased as well. Although Cd was transported to cucumber leaves more rapidly than Cu and Pb, Cu had a more toxic impact than Cd and Pb on photosynthesis. Barker (1972) studied toxicity levels of mercury along with other metals (Pb, Cu, and Zn) on tissue cultured lettuce, cauliflower, potato, and carrot. The study results showed that lettuce growth was inhibited by Hg concentrations of less than 0.005 mg/liter and at 5.0 mg/liter, the plant was almost dead and its tissue color looked gray. According to Santala and Ryser (2009), elevated heavy metal levels reduced plant size (birch seedlings) when they used three levels of Cu–Ni containing slag (0%, 0.5%, and 2.5%) mixed with sand in a growth substrate.

In addition to the effects on photosynthesis, enzyme activity, and oxidative membrane damage, the root elongation can be inhibited, (Shaw et al., 2004; Hartley et al., 1999). One of the notable effects of increasing heavy metals concentrations is reduction of root growth more than shoot growth (Santala and Ryser, 2009). In general, the root accumulates metals more than other parts of the plant, with an exception for hyper-accumulator plants (EPA, 2007). Root and shoot weights also decreased with an increase in Pb concentration (Verma and Dubey 2003).

Graber et al. (2009) used planted and unplanted trickling filters to treat contaminated wash water from a wood gasifier. They found that the yield of the

plants used in this system was similar to a conventional hydroponic system. However, the potential accumulation and the long-term effects of heavy metals in plants in hydroponic systems still need to be studied. Root and shoot are the two main structural components of plants. Roots absorb nutrients, and shoots (stem and leaves) convey minerals and water absorbed by roots to produce carbohydrates in leaves and then distribute oxygen to the root system (Shaw et al., 2004). Wang et al., (2018) examined the bioavailability of heavy metals at surfaces of root plasma membranes and the chemical forms of cells controlling nutrient transfer processes of plants. Metals may move to the root surface from the soil in two ways; either through concentration gradient of the diffusion or between the clay particles and the root by ion exchange (Shaw et al., 2004). The elements can move into the roots by several pathways such as passive diffusion through the root cell membrane, active transfer against concentration, or electrochemical potential gradients. Metal species can transport across the root membrane by binding with specific complexing agent like an organic acid or protein which latter dissociate into the plant cells (Shaw et al., 2004). It is assumed that heavy metals are taken up by specific proteins which function as transporters for essential elements (Lopez-Millan et al., 2009). This uptake process is dedicated to the essential trace metals, but at the same time, other metals can be taken up as well. Root elongation, seed germination, and water uptake of plants are common eco-toxicological measures for higher plants which can be used to evaluate and determine the phytotoxicity of toxic substances and particular compounds such as heavy metals in contaminated water like

wastewaters (Lyu et al., 2018; Song et al., 2017; Priac et al., 2017; Blok et al., 2008; Nagy, 2008; Di Salvatore et al., 2008; Ratsch and Johndro, 1986).

Several researchers evaluated the effects of heavy metals on the plant's growth, particularly on the root and shoot and mainly in lettuce (Lyu et al., 2018; Priac et al., 2017; Silveira et al., 2017; Park et al., 2016; Bautista et al., 2013; Cheung et al., 1989; Ratsch and Johndro, 1986). Monteiro et al. (2007), reported that accumulation of Cd concentrations in both roots and leaves increased over time, and the concentrations were higher in the root compared to the control at days 7 and 14 of exposure. Hartley et al. (1999) investigated the effects of soil contamination with single and multiple heavy metals including Cd, Pb, Zn, Antimony (Sb), and Cu on Scots pine seedlings colonized by ectomycorrhizal fungi. Root and shoot growth of the plant in a metal-amended soil were significantly inhibited. Cd was the most toxic to the symbiotic relationship of Scots pine seedlings and ectomycorrhizal fungi. Jadia and Fulekar (2008) studied the effects of some heavy metals (Cd, Pb, Zn, Cu, and Ni) on root/shoot growth of Sunflowers growing in soil-vermicomposting media that was inoculated with different levels of the metals (0, 5, 10, 20, 40 and 50 ppm) and to determine the impact on the root and the shoot growth. They found the metals effect increased at higher concentration. Xiong (1998) studied the bioaccumulation effect of Pb on the Chinese cabbage (*Brassica pekinensis* Rupr.). The results showed that root and shoot length inhibition depended on the Pb concentration; the length was shorter with the increase of the Pb. Also, the roots were more sensitive to Pb levels than the shoot. In a second study with *B. pekinensis* Rupr, Xiong et al.

(2006) evaluated the phytotoxic effects of Pb on Chinese cabbage (*B. pekinensis* Rupr.). The results showed Pb caused adverse effects on the growth of the plant; the shoot biomass decreased gradually when Pb increased in the plant shoot and the soil.

Di Salvatore et al. (2008) performed an experiment using two different germination substrates; agar and filter paper, to compare seed germination and root elongation and evaluate the toxicity of organic and inorganic compounds including metals such as (Cd, Pb, Ni, Cu) on various plants including lettuce. They found Cd produced the most toxic effects on plants, and lettuce was the most affected plant. Lettuce (*Lactuca sativa L.*) is a plant widely used in phytotoxicity studies (Silveira et al., 2017). In the present study, we hypothesized that heavy metals (Cd, Hg, and Pb), and a metalloid (As) would shorten the growth/elongation of root and shoot of lettuce growing in the aquaponic system after six weeks.

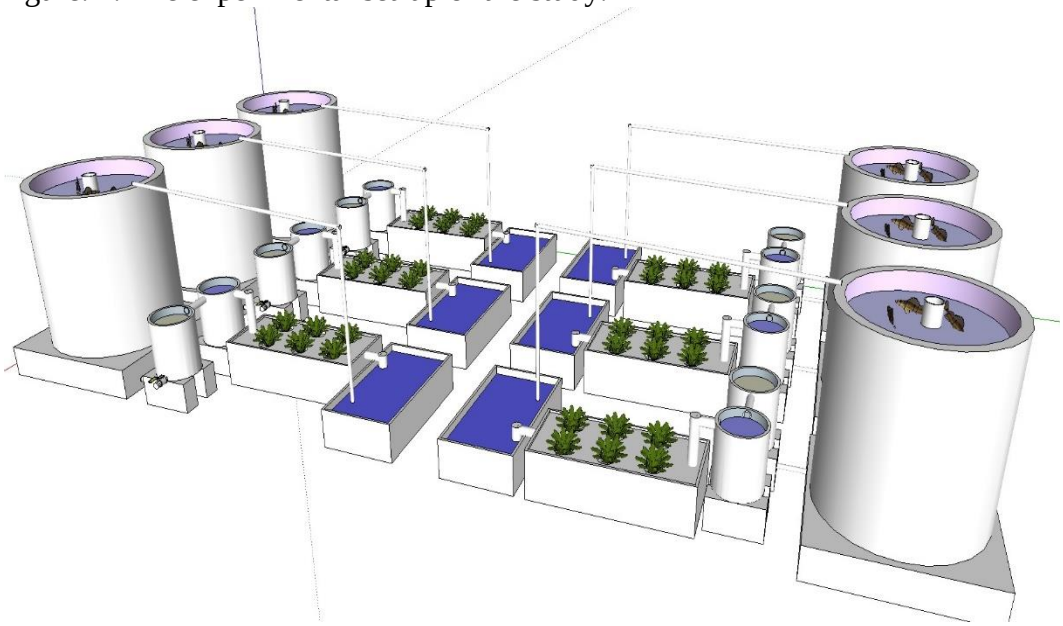
Material and methods

Experimental design

Six replicates were randomly distributed, three as control and three as treatment which were spiked with heavy metals and metalloid (HMs) (Fig. 1). Three heavy metals (cadmium (Cd), lead (Pb), mercury (Hg)) and one metalloid (arsenic (As)) were added into the aquaponic system with 25 fingerling Tilapia fish (*Oreochromis niloticus*) (25–50 g) provided by "Desert Springs Tilapia, Dateland, AZ, U.S. and six plants (Butterhead Lettuce-Rex pelleted seed (*Lactuca*

sativa) from Johnny's Seeds. The hydroponic system used in this experiment would commonly be described as deep-water culture (DWC). Tap water was used to start the experimental systems. The fish were fed a commercial diet (AquaXcel starter 5014 0.8 mm diet) with 50% minimum crude protein, 14% minimum crude fat, 2% maximum crude fiber, and 1% minimum phosphorus from Cargill Animal Nutrition. The experiment was conducted in a controlled environment (greenhouse) on The University of Arizona campus.

Figure. 1. The experimental set-up of the study.



Materials, Parameters, and instruments

Each replicate has two 20 L plastic containers functioning as a mechanical filter and a bio-filter. The mechanical filter included non-woven polyester fibers and was used for filtering suspended solids from the water and accumulated the sediments. The bio-filter was the site for the majority of bacterial activity including nitrification and heterotrophic decomposition. Both filters included

plastic bio-ball media used for increasing the bacterial surface area and enhancing their metabolic activity.

The fish were fed for two days in their tanks before the experiment started. During the trial, the fish were fed two times a day at 2.5% of biomass until the last day of the experiment. Fish were fed 2.5% of initial biomass for the entire trial. Each replicates system contained 244.1 L of water volume (65 L, 37.6 L, 25.2 L, and 115.8 L in the reservoir, the biofilter, mechanical filter, the grow bed, and the fish tank, respectively). Due to evapotranspiration, the replicates received additional deionized water into the reservoir.

A dissolved oxygen meter (Yellow Springs Instrument (YSI-550A) was used for measuring D.O. and water temperature (T.w). A water quality test kit from Hach Co. (DR/890 Portable Colorimeter) was used for measuring ammonia nitrogen (NH3-N) and nitrate ($NO^{-}3$), a portable pH meter from Hach Co. (HQ40d) for measuring pH with magnetic stirrer for more accurate results, and electrical conductivity meter (EC) from OAKTON Instruments for monitoring the total dissolved solids in the water.

Automated sensors in the greenhouse monitored relative humidity (RH), air temperature (Ta), and photosynthetically active radiation (PAR) (μmol·m-2·s-1). Other materials used included syringe filters (Nylon, 25mm diameter, 0.45um) from (Scientific Strategies. Co) and plastic syringe (Luer Slip, 10 mL) from (Materro LLC) for preparing water samples prior to submitting for HMs analysis. Rock-wool media was used for seed germination before placing in plastic hydroponic planting pots. A lab style drier was used for drying fish and

plant samples prior to HMs analysis. Air filter media (mesh) was used in the mechanical filter and a digital lab scale was used for determining weights of samples (wet and dry) and HMs prior to inoculation. A ceramic mortar and pestle was used for grinding the samples (fish and plants).

Chemicals (heavy metals inoculation)

At the beginning of the study, the fish feed and the water used in the experiment were submitted for analysis of HMs concentrations (Cd, Pb, Hg, and As) by the Arizona Laboratory for Emerging Contaminants (ALEC). HMs were spiked into the treatment replicates to reach molar concentrations (As, Cd, Hg, and Pb) 20%, 15%, 1.5%, and 1.5%, respectively, of the Maximum Contamination Levels (MCL) of the U.S Standards for drinking water (EPA, 2018). HMs were used in following forms: (As) as sodium hydrogen arsenate heptahydrate (Na_2HAsO_4), (Cd) as cadmium acetate dehydrate ($(CH_3CO_2)Cd{\cdot}2H_2O$), (Hg) as methylmercury chloride (CH_3HgCl), and (Pb) as lead (Pb^{2+}) acetate ($C_4H_{10}O_8Pb_3$). All HMs were purchased from Thermo Fisher Scientific Chemicals Inc.

Fish source and distribution

Three hundred juvenile Tilapia fish (*Oreochromis niloticus*) were provided by Desert Springs Tilapia, a commercial fish farm located in Arizona, U.S. The fish were acclimatized for two days and then the next day, six randomly selected fish (13 g total average ± 6 g) were removed and processed to determine the

background HM levels in the fish. The next day, 150 fish (13.5 g average) were weighed and randomly distributed into the replicates, 25 per system. Mortality was less than 5% of the population. Water temperature was controlled at 24°C during acclimatization.

Seedling and transplanting

80 seeds of Butterhead lettuce (*L. sativa* from Johnny's Seeds Co.) were germinated nine days before starting the experiment using rock-wool cubes which were cut from a slab using a plastic knife. The plants were irrigated with tap water daily for nine days) Six sample plants were collected for the HMs analysis to determine the background concentration. 36 plants were randomly selected for transplanting to the six replicates using plastic forceps on day one.

Fish feeding

Fish were fed two times daily based on percentage of the average body weight (2.5%) of all fish in each replicate. Therefore, 8.4 g of feed was added daily (half in early morning and the other half in afternoon). The feed ratio was based on the following formula:

Feed amount = Total fish biomass x 0.025

Replicate distributions

Each tank/replicate of the experiment was assigned randomly as control or treatment and labeled appropriately before starting (Fig. 1).

Water quality tests

Ammonia, nitrate, dissolved oxygen, water temperature, and electrical conductivity were determined for samples from each replicate at the beginning, at intermediate points, and at the end of the trial.

Ammonia

Ammonia concentration was determined as ammonia nitrogen (NH_3-N) with the Salicylate method 8155 (AOAC, 2018).

Nitrate

Nitrate concentration was determined as nitrate-nitrogen (NO_3-N), using the cadmium reduction method 8039 (AOAC, 2018).

Samples

Collecting samples and preparation

Six plants were collected randomly and separately from the original population (seeding tray) to determine baseline levels of HMs on day one. Plant (root and shoot) samples were collected and separated with each replicate treated in isolation from the rest of the replicates. After collecting the plant and cutting the shoots with plastic knives, the root was isolated from the shoot using plastic forceps and opening the rock-wool then carefully isolating the root. Then, length of roots and the largest/ most extended leaves of each plant were measured using a wood ruler. Then wet weight for each root and shoot sample were determined. All the samples of the root and the shoot were put on separate labeled Petri dishes before drying the samples in driers at 65˚C until they reached a

constant weight. Then, the dry shoot and root samples were ground using a mortar and pestle. The samples were then placed in separate sterile 50 ml sample tubes and homogenized by shaking the tube vigorously for 2 min and sent to the laboratory to determine the background HMs concentration of the roots and leaves tissue.

On the last day of the experiment, the same procedure from the first day was followed with some minor exceptions. The edible parts (the shoot for each sample) were put in a paper bag after determining the wet weight and the length of roots and shoots. Dry weight of roots and shoots were determined after removing samples from the dryer. Then, the dry samples were crunched by hand within the individual paper bags before using a stainless-steel grinder, then transferred into 50 ml plastic tubes.

All Petri dishes and plastic tubes were labeled with the details of the sample (sample code, treatment/control, replicate number, date of collection, and the collector name) prior to collecting and after the grinding step. Between each use ceramic mortar and pestle, and plastic forceps were washed with Diw then rinsed with nitric acid and wash again with Diw. Also, every shoot and root of each lettuce sample were cut using clean plastic forceps.

Laboratory analysis

All samples were sent to ALEC lab for analyzing. HMs were determined after extraction from the samples using inductively–coupled plasma mass spectrometry (ICP-MS).

Tissue sample preparation and digestion:

Well-ground tissue samples (< 100um) were oven-dried at 60°C and stored in a desiccator. The microwave-assisted acid digestion procedure (modified from US EPA Method 3051) is a closed vessel technique which uses 0.1 to 0.5g sample material plus 1mL concentrated nitric acid (Omni-trace HNO_3, EMD Chemicals), (1mL hydrogen peroxide (30%, Suprapure, EMD)) and 1mL ultrapure water (18Ω). Digestion is performed in MARS6 microwave digestion system (CEM Corp., Matthews, North Carolina).

Analysis by Inductively Coupled Plasma Mass Spectrometry

Solutions were analyzed on an Agilent Model 7700x ICP-MS (Santa Clara, CA). Instrument parameters used are listed in Table. 6.
Before initial calibration, a daily performance check served to verify instrument response over the mass range from Li to Tl, to monitor background noise level and the presence of oxides (CeO/Ce) and doubly-charged ions (Ba^{++}/Ba) which must be less than three percent.

Calibration standards were prepared from multi-element stock solutions (except for Hg, which is a single element standard) purchased from AccuStandard (New Haven, CT).

The stocks were diluted in 1% nitric acid to provide a working calibration curve of at least 5 points. Samples were also diluted with 1% nitric acid until their response was determined to be within the calibration range. Internal standards (Rh, In and Ga) were added to both standards and samples before analysis using a mixing tee in the sample introduction system.

Quality Control measures

Following the US EPA protocol in Method 6020, each run included quality control checks referred to as Initial Calibration Verification (ICV) standards and Independent Calibration Verification. These QC checks must fall within +/- 10% of their expected value.

A mid-range standard was analyzed after every ten samples and again at the end of the run. These QC checks were referred to as Continuing Calibration Verification (CCV) samples, and the results must fall within 25% of the expected value.

Also, a QC solution sample (such as NIST 1643e Trace metals in water) was chosen to match the matrix of the samples to be analyzed and was included at the beginning and end of each sample set.

Safety precaution

The laboratory and work areas were always kept clean and uncluttered. Personal Protective Equipment (PPE) were worn at all time while dealing with the chemicals or the samples. The PPE included goggles, gloves, appropriate shoes, long-sleeved clothes, and lab coat. Gloves were changed whenever dealing with samples of a new replicate. All precautions stipulated in laboratory protocols material safety data sheets were followed. All hazardous materials and the remaining HMs solutions were kept in a special container provided by the Department of Risk Management Services (RMS) of the University of Arizona for later disposal. The work area was properly-ventilated when dealing with the heavy metals and the nitric acid.

Statistical analysis

Six replicates are obtained represented the results of the study. Statistical comparisons were performed between treated and control of the plants using Ttest; Average of the plant results (n=18) of the treatment compared with the plants of the control. Results are expressed as mean ± SD. $P<0.05$ considered as a significant probability.

Results

Parameters

During the study period, environment parameters were in normal range for the air temperature, the relative humidity, and the photosynthetic active radiation were 22.2 C°, 44.81%, and 43.4 μmole/m^2/s, respectively from Jan 8th (day 1) to Feb 12th (day 35) of 2018. Also, the range of the pH was 8.2-7.0, the DO was 6.5-5.8 mg/l, the EC from 0.4-0.7 mS/cm f, the Tw were 23.6-26 C°, the ammonia (NH_3) was 0.05-0.25 mg/l, and the average of nitrate (NO_3) was 3.1-22.2 mg/l.

The effect of the HMs concentrations on the lettuce growth evaluated by measuring the root and the shoot elongation and the dry weight of the plant (plant mass) for both the control and the treatment. The length of the roots and the shoot and the dry weight of both of them were reported as the following results:

The root length

The concentrations of the HMs tested did not have an effect on the length of the lettuce roots of the treatment compared to the control (Fig. 2); there was no difference between the treatment compared to the control ($P > 0.05$) (Table. 1).

Figure. 2. The average length of the lettuce roots for the control and the treatment of the last day with the standard deviation.

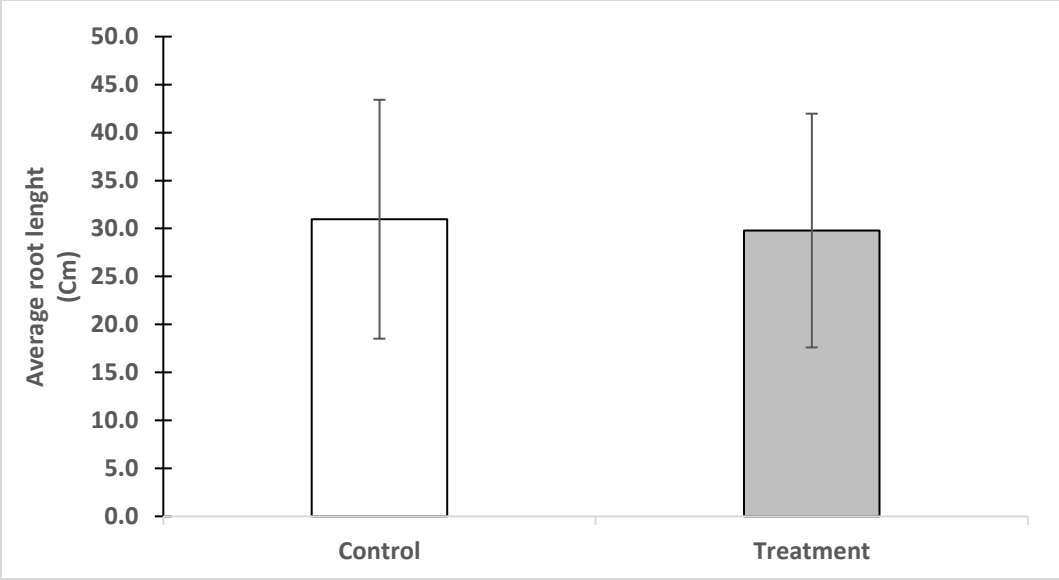


Table 1. The total length of the lettuce roots for the control and the treatment of the first and the last day with the standard deviation and the P-value (the means of the control and the treatment).

Average root length (cm)					
Treatment	**First day**	**S.d**	**Last day**	**S.d**	**P value**
Control 1	8.75	3.71	18.85	7.56	0.8
Control 2	8.75	3.71	34.00	7.41	
Control 3	8.75	3.71	40.05	10.69	
Treatment 1	8.75	3.71	18.97	5.76	
Treatment 2	8.75	3.71	37.70	8.50	
Treatment 3	8.75	3.71	32.70	12.31	

The shoot length

Compared to the control, the treatment results show no effect of the HMs concentrations on the shoot length ($P > 0.05$) (Fig. 3) and (Table. 2).

Figure. 3. The average length of the lettuce shoots for the control and the treatment on the last day. (± standard deviation)

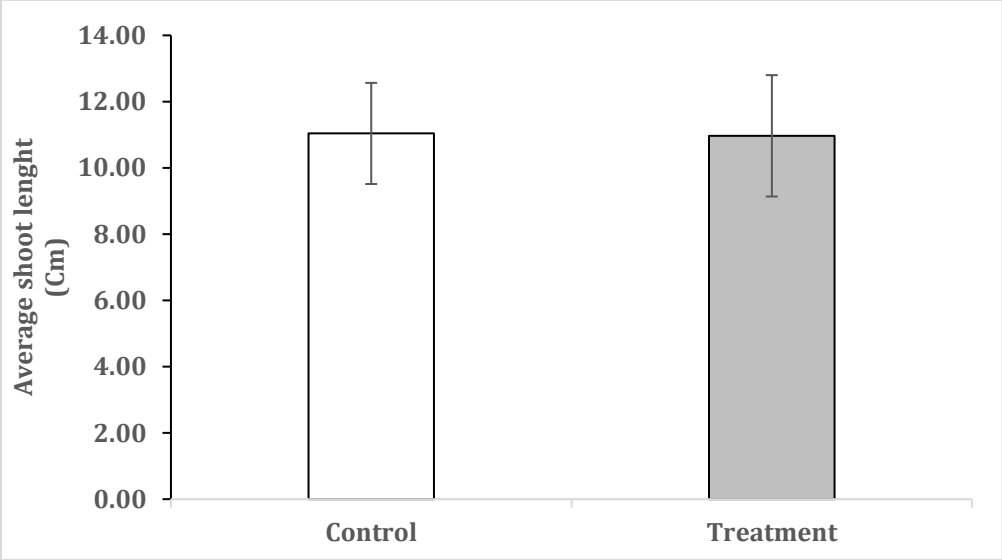


Table 2. The total length of the lettuce shoots for the control and the treatment of the first and the last day with the standard deviation and the P-value (the means on the control and the treatment).

Average shoot length (cm)					
Treatment	**First day**	**S.d**	**Last day**	**S.d**	**P value**
Control 1	2.28	0.71	9.33	0.51	0.9
Control 2	2.28	0.71	11.87	1.11	
Control 3	2.28	0.71	11.92	1.07	
Treatment 1	2.28	0.71	9.18	1.41	
Treatment 2	2.28	0.71	11.58	0.98	
Treatment 3	2.28	0.71	12.13	1.49	

The root growth (dry weight)

The results of the dry weight of the root of the treatment compared to the control show no effect of the HMs tested on the growth of the plant ($P > 0.05$) (Fig. 4) and (Table. 3).

Figure. 4. The average dry weight of the lettuce root for the control and the treatment at the end of the study. (±standard deviation)

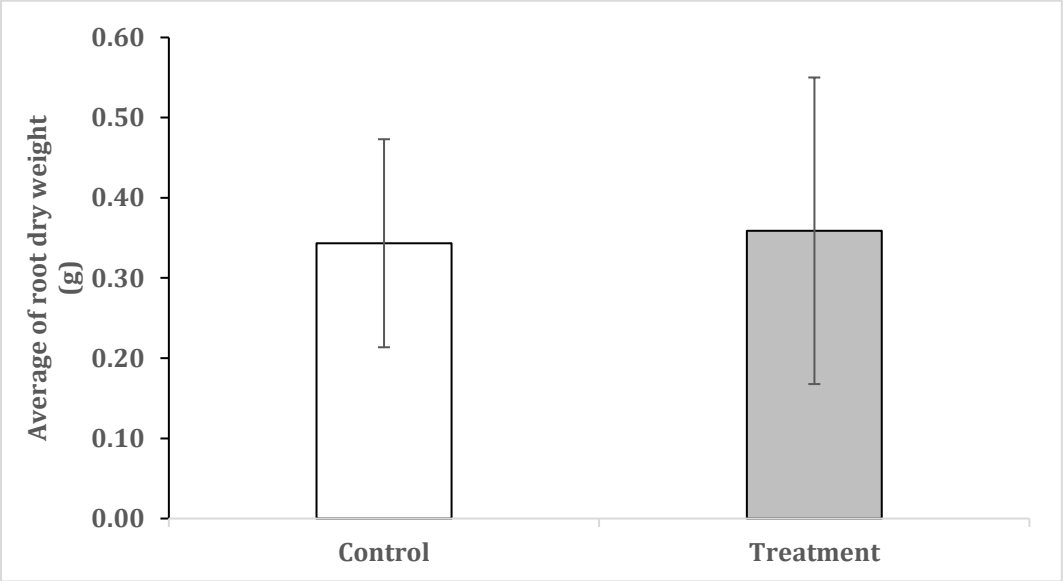


Table 3. The total dry weight of the lettuce root for the control and the treatment at the end of the study with the standard deviation and P-value (the means of the control and the treatment).

Total dry weight of the root (g)			
Treatment	**Dry weight**	**S.d**	**P value**
C1	0.29	0.07	0.8
C2	0.36	0.09	
C3	0.39	0.18	
T1	0.24	0.11	
T2	0.51	0.19	
T3	0.33	0.16	

The shoot growth (dry weight)

The HMs tested did not affect the shoot growth; no difference reported by the last day of the experiment between the treatment and the control ($P > 0.05$) (Fig. 5) (Table. 4).

Figure. 5. The average dry weight of the lettuce root for the control and the treatment at the end of the study. (±standard deviation)

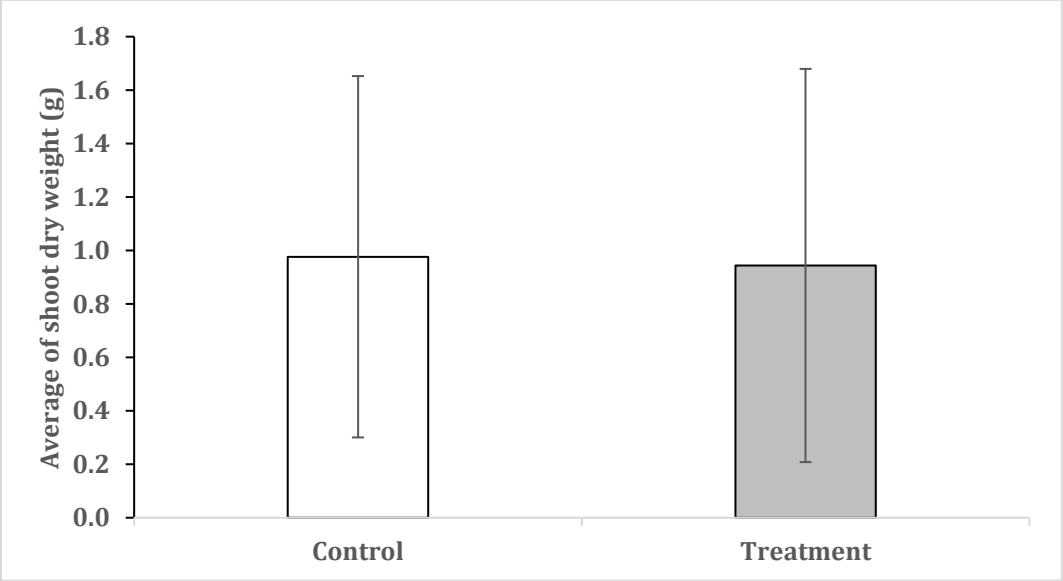


Table 4. The total dry weight of the lettuce shoot for the control and the treatment at the end of the study with the standard deviation and P-value (the means of the control and the treatment).

Average shoot dry weight (g)			
Treatment	**Dry weight**	**S.d**	**P value**
Control 1	0.72	0.37	0.9
Control 2	1.37	0.75	
Control 3	0.82	0.65	
Treatment 1	0.64	0.65	
Treatment 2	1.16	0.65	
Treatment 3	1.02	0.79	

Discussion

Elevation of metal ions can inhibit the elongation of plant roots (Santala and Ryser, 2009) and shoots (Cheung et al., 1989). One of the notable effects of increasing heavy metals concentrations is reducing root growth more than shoot growth (Santala and Ryser, 2009). In general, the root of the plant accumulates more of the heavy metals with an exception for hyper-accumulator plants (EPA,

2007). Reducing root growth more than shoot growth is a common effect associated with elevated concentrations of heavy metals (Santala and Ryser, 2009).

Root elongation inhibition has been used as a measure for determining various toxic substances such as heavy metal in plants (Ratsch and Johndro, 1986). In this study, the root and the shoot elongation of the lettuce as well as the plant mass (dry weight of the treatment and the control) were evaluated as an indicator of the potential toxic effects of the tested HMs that accumulated (from spiking, from background in tap water and leaching or metabolized from feed) in the aquaponic system.

The concentrations of the HMs tested in our experiment did not disrupt the root and the shoot length nor the growth of the dry weight/mass of the lettuce. The results of the average root length (Fig. 2) show there was no effect of the HMs at the level tested on the root length of the plant ($P > 0.05$) (Table. 2), 31 cm and 29.8 for the control and the treatment, respectively, and the results of the average root mass (average root dry weight) were also not significantly different ($P > 0.05$) (Table. 4), (0.34 g) of the control and (0.36 g) of the treatment (Fig. 4).

The lengths of the leaves were similar between the control (11.04 cm) and the treatment (10.97 cm) (Fig. 3); therefore, the lengths of the lettuce shoots were not affected by the increased HMs (the treatment) ($P > 0.05$) (Table. 2). Also, as reported in the root mass, the average dry weight of the shoots demonstrated similar growth between the control (1.0 g) and the treatment (0.9 g) (Fig. 5), and there was no difference between them ($P > 0.05$) (Table. 4).

In general, the levels of the HMs in our study showed the metals did not exhibit any significant effect either on the root length nor the plant mass. Lettuce is considered as one of the potential hyper-accumulators plants for heavy metals which can accumulate metals in their tissues without showing any toxicity (Khan et al., 2015). There are several studies which evaluated the effect of different metals at various levels on crops, including lettuce, with different cultivation methods or media types. Bautista et al. (2013) evaluated the effect of absorbing different concentration (25 µM, 35 µM, and 50 µM) of Cd and Cr on root elongation of lettuce and other plants (Swiss chard, and spinach) after germination of the seeds on filter papers. Cd had a greater effect on root elongation than Cr, with lettuce being affected the most. Monteiro et al. (2007) evaluated the genotoxic effects of Cd in lettuce (*Lactuca sativa* L.) at cell level after growing five-week-old of the plants in perlite media with concentration at 100 µM of $Cd(NO_3)_2$ (water-dissolve form). The Cd accumulated in the root (2.167 mg/g) and the shoot (0.344 mg/g) (as dry weight) over the time of Cd-exposure (14 days); the root accumulated eight-fold higher levels on the same day. The data exhibited an inhibition of the lettuce growth; the shoot length was reduced significantly but was not significant for the root length compared to the control. However, we have tested smaller concentrations of Cd, the media used for the cultivation and the time they used for inoculating Cd were different, and we tested a mixture of metals in an aquaponics system, but not hydroponic. In our study, there was no significant difference of the treatment replicates between the root and the shoot lengths. Although we also had higher Cd concentrations in the root

of the lettuce compared to the shoot, but there was no effect of the metal on the length or the mass of the plant during our time frame.

Xiong et al. (2006) found that increased concentration of Pb for treatment levels in Chinese cabbage (*Brassica pekinensis Rupr.*) did not induce visible toxic symptoms, but shoot length was significantly shorter with the increase of Pb concentrations in the soil. The shoot biomass was less than the control and the plant accumulated more of the metal when the metal concentration was higher in the soil. Although Pb increased significantly ($P < 0.05$) in the root of the lettuce in our study, as we mentioned, the plant mass was not affected.

Verma and Dubey (2003), studied the effect of different levels of Pb after seedling rice in sand culture, and they found Pb bioaccumulated more in the roots than the shoots, and both root and shoot lengths, as well as their weights were diminished with the increasing concentration of Pb.

Jadia and Fulekar (2008) reported the toxicity of metals increased proportionally with their concentrations in the soil which showed a significant reduction on the root and shoot growth of sunflowers. On the other hand, the lower concentration of Pb stimulated the biomass of the sunflower and increased the root and shoot length. Chaves et al. (2011) evaluated effects some heavy metals (Cd, Cu, and Zn) concentration on the growth of sunflower plants in soil examining their uptake into the plant tissues. Cd was applied into the soil with different rates (0, 10, 20, 30, and 40) mg/dm^3. The high concentrations of the metals impacted the growth of the plant, but, no significant effect was reported on the plant dry matter. The high concentration of Zn caused a significant decrease in

leaf area and also a significant accumulation effect in the stem, leaves, and roots of the sunflower. On the other hand, shoot dry matter slightly increased at low concentrations (10 mg/kg of Cd) and (20 mg/kg) of Cu and Zn compared to control.

It has been reported that heavy metals at high concentrations can reduce plants elongation and their growth, and delay cell division (Chaves et al., 2011). Although several studies showed significant effects of the heavy metals on the length of the root and the shoot and also on the biomass of the plants, our studies did not demonstrate significant differences. In addition to the low concentrations tested in the current study, there could be two potential factors that may have impacted the present results, the defense mechanism of the plants root and the possible effect of the interactions of the different HMs we tested.

Plants release border cells from the root cap to confront potential hazards such as microbial infection or contaminants like heavy metals in the surrounding environment (Tran et al., 2016; Tollefson et al., 2015). As produced in most plants, root tip extracellular matrix includes border cell populations (Huskey et al., 2018). The root border cells are attached structures that cover plant roots tips like a sheath that surrounds root tips. Similar to neutrophils in mammals, border cells provide protection to the root by producing an extracellular matrix of proteins, polysaccharide and external DNA (exDNA) The exDNA released by the border cells disperse into the environment of the plant roots and possibly defend them by trapping invaders such as root pathogens or metals which can be then immobilized (Huskey et al., 2018; Hawes et al., 2016; Tran et al., 2016; Tollefson

et al., 2015; Curlango-Rivera et al., 2014; Driouich et al., 2013). Border cell slime layers can be induced within minutes where they can trap metals such as Al, Cu, and others within the matrix. The gene expression of these detached root border cells on the root cap has a higher rate of metabolic activity than their ancestor cells (Tollefson et al., 2015).

In addition to the previous reasons, competing and interacting each of the tested HMs with the bioavailability and concentration of essential and nonessential metals in the water may lead to impaired absorption of the HMs by the plants. These toxic metals can interact thus their toxicity can be decreased in the plant tissue, particularly in root tissue, such as Cd with Pb (Lepp, 1977). Weakening the toxicity effect of the HMs tested in the system is a potential factor that may be involved with the plant growth.

It is known that even the essential metals can be toxic if present in excess. An excess of essential metals such as Cu may inhibit root growth and damage the plasma membrane resulting in ion leakage from the cells (Bouazizi et al., 2010). Di Salvatore et al. (2008) evaluated the toxicity of some metals (Cd, Pb, Ni, and Cu) from 0 to 1024 µM on the seed germination and root elongation of different plants (lettuce, broccoli, tomato, and radish) using two different growth media: agar and filter paper. They found that the agar test is more sensitive than that on filter paper; while germination is not affected by media of the growth, root length is affected by the increasing concentrations of the metals. Cd was the most toxic metal on the different plants; it showed the highest toxicity on broccoli and lettuce. Among the plants tested on the agar, lettuce was the most effected plant.

As mentioned in the study of Monteiro et al. (2007) when Cd (100µM of $Cd(NO_3)_2$ accumulation increased in roots and leaves of lettuce (*Lactuca sativa*) due to the exposure time (14 days in a growing media) after growing five-week old plants. However, neither Cd nor the other HMs tested in the current study accumulated in the root or the shoot, except for Pb and As where they accumulated significantly in the root by the end of the study period (35 days).

More studies may be needed to evaluate the potential effect of the accumulation of heavy metals on plant growth in aquaponics, particularly, by continuing the system for longer period with several cycles, and maybe at higher concentrations levels. Future work may also include additional experiments to evaluate the effect of each of the HMs separately. Lepp (1977) studied the effect of single or dual applications of several heavy metal ions (Cd, Pb, Cu or Ni) on seedlings of lettuce. He found that metals show different interaction between each other. Applying these metals at (10 µg/L) showed significant reductions in both roots and shoots growth of the plant while there was no significance when the dual metals were applied at the same concentrations.

Conclusion

The concentrations tested for the HMs in the present study did not cause any effect on root elongations or shoot length. The plant mass (dry weight) results showed no significant differences between the treatment and control.

TABLES

Table 5. The total length (cm) of the roots and the shoot and dry weight (g) of the lettuce for the control and the treatment at the last day of the experiment.

Treatments	Replicate #	Length (cm)		Dry weight (g)	
		Root	Shoot	Root	Shoot
Control #1	**1**	17.2	9.4	0.33	1.1
	2	17.5	8.8	0.13	0.2
	3	15	8.9	0.29	0.7
	4	16.9	10.1	0.36	1.2
	5	11.4	8.9	0.29	0.3
	6	35.1	9.9	0.31	0.8
Control #2	**1**	30.9	11.1	0.31	0.8
	2	35.2	11.2	0.37	1.2
	3	43.2	14.2	0.5	2.7
	4	43.5	12.2	0.43	2
	5	24.6	11.2	0.24	0.5
	6	26.6	11.3	0.31	1
Control #3	**1**	25.2	11.2	0.23	0.2
	2	30.9	13.4	0.4	0.4
	3	51.1	13	0.72	X*
	4	47.4	11.9	0.32	1
	5	52.6	10.2	0.17	0.5
	6	33.1	11.8	0.47	2
Treatment #1	**1**	13.6	8.6	0.14	0.3
	2	10.1	7.1	0.13	0.1
	3	26.1	9.9	0.23	0.9
	4	17.6	8	0.18	0.1
	5	21.9	11.2	0.32	1.8
	6	24.5	10.3	0.43	X*
Treatment #2	**1**	47.3	12.4	0.56	X*
	2	32.9	11.5	0.34	1.2
	3	49.2	13.3	0.81	2.4
	4	27	10.9	0.29	0.6
	5	29.6	10.9	0.38	0.8
	6	40.2	10.5	0.67	0.8
Treatment #3	**1**	41.4	13.1	0.35	1.5
	2	21.9	10.3	0.17	0.2
	3	26.7	12.2	0.32	0.8
	4	56.3	13.8	0.64	2.5
	5	23.7	13.4	0.34	0.8
	6	26.2	10	0.16	0.3

X*: The data excluded due to incomplete drying.

Table. 6. Instrumentation parameters for ICP-MS (provided by ALEC laboratory)*.

RF power (w)	1450
Dwell time (ms)	50
Sweeps per replicate	100
No. of replicates	3
Acquisition mode	Peak hopping
Argon flow rates (L/min):	
Nebulizer flow	0.95
Coolant	15
Auxiliary	1.3
Sample uptake (ml/min)	~0.400
Presence of oxides as CeO/Ce	< 3%
Presence of doubly- charged species (as Ba^{++}/Ba)	< 3%
Nebulizer type	Micro-mist
Spray chamber	Scott Double-pass quartz
Sample and Skimmer cones	Ni

*provided with permission from ALEC lab, University of Arizona, Tucson, Az, U.S.

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